THE CONTRIBUTIONS OF THE *OMPF* AND *OSMB* GENES IN PROMOTING *YERSINIA PSEUDOTUBERCULOSIS* SURVIVAL DURING DOXYCYCLINE EXPOSURE

by
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Abstract

Antibiotic-resistant bacterial infections pose a major public health threat. The current rise in antibiotic resistance is potentially leading to a post-antibiotic era in which antibiotics may no longer be useful in treating many bacterial infections. Within bacterial populations there exist subpopulations of antibiotic-tolerant cells defined as persisters, or persistent cells, whose reduced metabolic activity and slowed growth rates reduce their susceptibility to antibiotics. Persisters are known to harbor and spread antibiotic resistance genes, which contribute to the threat of antibiotic resistance. Exposure to antibiotics has been shown to promote persistence, however, few studies have characterized persister cell formation within host tissues.

In this thesis, the role of clinically relevant doses of antibiotics in the induction of persistence is explored in *Yersinia pseudotuberculosis.* It is shown that Osmotically-inducible lipoprotein B(OsmB) and Outer membrane porin F (OmpF) play a role in the accumulation of antibiotics within bacterial cells. Expression of these gene products, however, did not significantly impact the susceptibility of *Y. pseudotuberculosis* to doxycycline.

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Introduction

Antibiotic-resistant infection is an ever-growing threat to public health. There are approximately 2.8 million cases of antibiotic resistant infections in the United States each year according to the Centers for Disease Control and Prevention (1). Antibiotic resistant infections are difficult to treat and not only lead to increased morbidity and mortality but also cost roughly $4.6 billion a year to treat (2). The World Health Organization has listed antimicrobial resistance as one of the top ten threats to global public health (3).

There are several reasons bacterial infections may survive within a host following antibiotic treatment. The bacteria causing the infection may be resistant to the antibiotic being used to treat the infection (1, 4-7). The bacterial population may be tolerant to antibiotics and regrow after treatment (7-12). The bacteria may be in sites such as deep tissues that are not easily accessible to antibiotics. Or lastly, subpopulations of bacteria within the infection may have reduced susceptibility to antibiotics (2, 13-15).

Persistent bacteria, known as persisters, are a subpopulation of bacteria that are less susceptible to antibiotics and remain after antibiotic therapy. Persistent bacteria are known to cause recurrent infection and drive resistance (14). Persistent bacteria have been called a catalyst for the increase of antibiotic resistance due to their ability to serve as a reservoir of antibiotic resistance genes that can be passed on to other bacteria (16). The identification of potential therapeutic targets to eliminate persistent bacteria within an infection would allow for more effective treatment of these infections and reduce the rate at which antibiotic resistance is spreading.

Persisters arise naturally during bacterial infection and can survive antibiotic treatment even when the majority of the bacteria in the population are susceptible to antibiotics (17).

Persistence is most often the result of corrupted central metabolism, however there are diverse stressors that can lead to this phenotype (18, 19). To investigate the potential role of antibiotics as a stressor leading to persistence, a *Yersinia pseudotuberculosis* model was used. Doxycycline is an antibiotic commonly used to treat *Yersinia* infections in humans and animals, and in a mouse model of infection, *Y. pseudotuberculosis* is able to survive treatment with doxycycline (7). It is unclear what allows for this survival. Doxycycline accumulation may lead to adaptations by the bacteria that protect them from antibiotic treatment. When exposed to doses of doxycycline between 10ng/ml and 100ng/ml, the expression of outer membrane proteins OsmB, a lipoprotein, and OmpF, a porin, is increased (20, 21).

To determine if the adaptation caused by doxycycline promotes antibiotic persistence during *Y. pseudotuberculosis* infection, deletion strains for the genes *osmB* and *ompF* were tested to measure their antibiotic susceptibility and accumulation *in vitro*. A *Y. pseudotuberculosis* mouse model of infection was also used to examine if *osmB* and *ompF* affect antibiotic susceptibility *in vivo*.

Antibiotics

Antibiotics can be broken down into two general categories: bactericidal, those antibiotics that directly kill bacteria; and bacteriostatic, those antibiotics that restrict bacterial growth. The activity of an antibiotic may change depending on the conditions in which it is used and the bacteria against which it is being targeted (22). At sufficiently high concentrations some bacteriostatic antibiotics will cause cell death. It is not clear, however, how this happens in all cases.

For any antibiotic to be effective it needs to be able to penetrate a cell through porins or by diffusion through the cell membrane. The antibiotic then needs to be able to bind to a

cellular target to either kill the bacteria or arrest its growth. Bactericidal and bacteriostatic antibiotics target aspects of bacterial physiology that are unique from human cells such as the cell wall, protein synthesis, and nucleic acid synthesis (4).

Bactericidal antibiotics such as β-lactams can directly kill bacteria by inhibiting cell wall synthesis. β-lactams bind penicillin-binding protein to prevent cross linking of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM), which compromises the integrity of peptidoglycan that makes up the bacterial cell wall (Figure 1.). Disruption of the cell wall can then lead to cell death. Death in this case results from lysis of the bacteria as the compromised cell wall is unable to withstand osmotic stress (22).

Bacteriostatic antibiotics in general inhibit some aspect metabolism to prevent growth. Tetracyclines are a family of bacteriostatic antibiotics commonly used to treat both human and animal infections. Tetracyclines act by binding the 30S ribosomal subunit of the bacterial ribosome (Figure 1.). Bacterial ribosomes present an attractive target for antibiotics because they have significant differences from eukaryotic ribosomes. Binding of the 30S subunit by tetracyclines prevents charged tRNA from entering the ribosome and inhibits protein synthesis. The mechanisms that lead to clearance of an infection after treatment with bacteriostatic antibiotics remain a matter of debate. However, clearance may be mediated by the host immune response or cell death caused by the accumulation of toxic metabolites leading to DNA damage in the bacteria (22).

Antibiotic Resistance

Antibiotic resistance is caused by specific resistance genes or mutations that prevent the activity of an antibiotic and significantly increase the minimum inhibitory concentration (MIC) of

antibiotic needed for activity. The MIC is the minimum concentration of antibiotic at which bacteria are killed or cannot grow (4).

Antibiotic resistance can be caused by multiple mechanisms such as antibiotic-degrading enzymes, efflux pumps, and alterations to the targets of antibiotics (Figure 1.). The genes that encode these mechanisms can commonly be found on mobile genetic elements such as plasmids, transposons, and phages, which allow antibiotic-susceptible bacteria to rapidly acquire resistance genes (4, 17, 23, 24). These genes are heritable, all daughter cells will have a resistant phenotype. Antibiotic resistance genes may not be ubiquitous in a population at the initiation of an infection, however, treatment will select for this population and lead to the entire population being resistant.

It has been demonstrated that genes that encode efflux pumps or antibiotic degrading enzymes were present in bacterial populations before the widespread use of antibiotics (25). This is because bacteria and other microorganisms produce antimicrobial compounds to out-compete other bacteria or fungi. To counteract such antimicrobial compounds, bacteria developed resistance mechanisms (26). Many antibiotics in use today were first identified in microorganisms such as bacteria and fungi (22). The increased use of human-derived antibiotics to treat bacterial infection and widespread antibiotic use in agriculture have created selective pressure leading to the rapid emergence and spread of antibiotic resistance (27).

Common Antibiotic-Resistant Infections

Many antibiotic-resistant infections are acquired in hospitals (nosocomial infections) and can lead to prolonged stays and worsened outcomes for patients who are often already in poor health. Ventilator-associated pneumonia (VAP) and *Clostridioides difficile* infections are both dangerous infections that are acquired in a hospital setting. Antibiotic resistance is

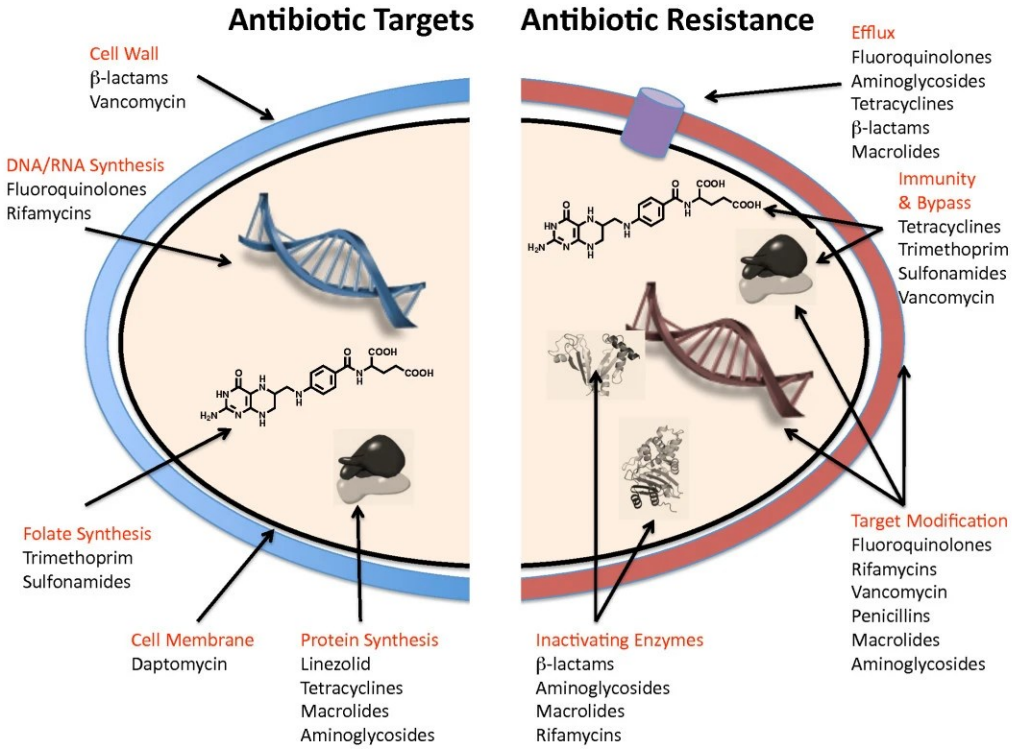


Figure 1. Antibiotic targets and resistance mechanisms. (Adapted from Wright, 2010)
Common targets for antibiotics are identified on the left side of the image along with major families of antibiotics that use these targets. Antibiotic resistance strategies utilized by bacteria to escape killing by antibiotics are listed on the right along with families of antibiotics that are affected by these resistance mechanisms.

frequently reported in bacterial VAP and *C. difficile* infections complicating the treatment for these infections.

Ventilator-associated pneumonia (VAP), caused by multiple genera of bacteria such as *Enterococcus* and *Staphylococcus,* is the most common nosocomial infection acquired in intensive care units. *Enterococcus* and *Staphylococcus* can be multi-drug resistant and the site of VAP being in the lungs can further limit antibiotic diffusion and make these infections difficult to clear. During the SARS-CoV-2 pandemic there has been a reported increase in VAP and worsened outcomes in patients with both COVID-19 and VAP (28). In some settings the rates of drug resistance in VAP can range between 40-80%, making the treatment more difficult for patients who are already intubated (29).

Clostridioides difficile is another dangerous antibiotic-resistant nosocomial infection. This infection is often caused by treatment with antibiotics for other infections and can arise from dysbiosis after the normal gut microbiome is altered by antibiotics (30). *C. difficile* is present in the gastrointestinal tract of many individuals, however, it is usually unable to out-compete other members of the microbiome (31). When broad spectrum antibiotics are used to treat bacterial infections or during surgical procedures, large populations of bacteria in the intestinal microbiota are killed off allowing for the antibiotic resistant *C. difficile* to replicate to dangerous numbers, which can lead to debilitating diarrheal disease and recurrent intestinal infections.

The threat of antibiotic resistance also extends outside of the hospital setting. Many pathogenic bacteria, such as *Mycobacterium tuberculosis* and *Neisseria gonorrhea* have acquired antibiotic resistance, and these infections are more commonly community acquired. *M. tuberculosis* infections require long-term treatment ranging from six months to years with

multiple antibiotics such as rifampin, isoniazid, and fluoroquinolones (32). This treatment has further been complicated with the emergence of multi-drug resistant and extensively drug resistant *M. tuberculosis*. Multi-drug resistant *M. tuberculosis* (MDR-TB) is resistant to rifampin and isoniazid, and extensively drug resistant *M. tuberculosis* (XDR-TB), which are MDR-TB strains that are resistant to fluoroquinolones and second line antibiotics (33). The World Health Organization reported that in 2016, 240,00 people died from infections with MDR-TB and a 4.1 per cent increase in cases. Rising rates of drug resistance in *M. tuberculosis* is a global threat that will continue to lead to more deaths (34).

Antibiotic Persistence

All bacterial populations are heterogeneous. One aspect of this heterogeneity is the presence of subpopulations of bacteria that stochastically arise that manifest reduced metabolic activity and slowed growth rates (35). These slow-growing cells are defined as persistent cells, or persisters (36). Reduced metabolism is strongly linked to persistence during antibiotic treatment (37). Antibiotics do not act effectively on these cell populations, and persisters will remain following antibiotic treatment. It is thought that these persistent bacteria arise within the population at a set rate, with that rate varying greatly from species to species, and even from strain to strain within a species (35). The presence of persistent bacteria can prevent effective clearance of the infection and lead to recurrent infections.

Research has shown that the presence of persistent cells in a bacterial infection can be a driver of the spread of antibiotic resistance. Persisters can lead to antibiotic resistance through several mechanisms. The same pathways that can induce persister cell formation, such as the SOS response, can also lead to an increased rate of mutations, which can alter the target of antibiotics (38). The SOS response is the bacterial response to DNA damage that has diverse

effects on the cell many of which lead to tollerace. Persisters can also serve as a reservoir for mobile genetic elements that contain antibiotic resistance genes (39). The stressors that lead to persistence also cause the activation of these mobile genetic elements spreading antibiotic resistance genes to previously susceptible populations of bacteria.

When an infection containing persistent bacteria is treated, the majority of the population will be killed off, leaving a persistent subpopulation that can regrow after treatment. Clearance of persistent bacteria is important to fully treat an infection and avoid recurrent infections. The reduced activity of antibiotics against metabolically inactive persisters is generally thought to result from the reduced availability of antibiotic targets. Many antibiotics target bacterial processes such as protein synthesis and cell wall synthesis that are most active during growth. Reduced activity of these pathways can result in fewer targets for antibiotics. Antibiotics in the β-lactam family, for example, target cell wall synthesis, and without active synthesis they do not kill bacterial cells (40). Reduced metabolism may also limit the targets for other antibiotics. Lower ribosomal content in less active cells could potentially limit the efficacy of doxycycline and other tetracycline derivatives that target the ribosome (4).

Persistent bacteria do not require antibiotic resistance genes to survive antibiotic treatment, but it is thought that there is a genetic basis for the switch to a persistent phenotype. Persistence is a phenotypic change that can be induced by reduced metabolism and dormancy. Dormancy is a state of non-division that allows bacteria to survive harsh environments. The signaling that initiates phenotypic switch to slowed metabolism or dormancy is genetically encoded. This has been shown through the enrichment of persister bacteria within a population after multiple brief rounds of antibiotic treatment (16). Altering the rates of persister formation is thought to allow bacterial populations to respond to changes in their environment by increasing the rate of persister formation in harsh conditions or reducing the rate of persister

formation in more hospitable conditions. The occurrence of persisters in bacterial populations has been proposed to be a bet-hedging strategy where the bacterial population sacrifices some potential for growth to be able to survive harsh conditions such as low pH, desiccation, and exposure to antibiotics. There are also other theories that explain persistence as arising from random cellular errors that lead to growth arrest. The genetic foundation of bacterial persistence is not yet well understood (41). There are currently very few drugs that directly target persistent infections, which are generally treated with short treatments of high doses of antibiotics or long treatments with low antibiotic doses. High-dose interval treatment has been shown *in vitro* to increase the percentage of the population that is persistent, making these infections particularly difficult to treat (16).

It is difficult to directly measure the impact of persistence. Detecting persistent cells once the bacteria is cultured from a patient is difficult because the bacteria are no longer exposed to the stressors that led to persistence in the host. These infections may not be culturable because they are metabolically inactive or dormant, further complicating diagnosis. Measuring antibiotic persistence is more complicated than measuring antibiotic resistance, which can be directly measured at the population level by calculating the MIC and is not dependent on a stress induced phenotype. The reversal of the persistent phenotypes when removed from stressors and the potential to be nonculturable make the rapid diagnosis and surveillance of the presence of persistent cells more complicated than surveillance of resistance. The complicated nature of surveillance of persistent infections makes it difficult to fully assess the impact of persistent infections, and can led to the misidentification of persistent infections as resistant infections (14).

Common Antibiotic-Persistent Infections

There are many common bacteria that can establish persistent infections including *M. tuberculosis*, *Salmonella typhi*, *S. aureus*, ureopathogentic *Escherichia coli* (UPEC), and *Pseudomonas aeruginosa* in patients with cystic fibrosis. The most prevalent example of a bacterial pathogen that establishes persistent subpopulations is *M. tuberculosis*. *M. tuberculosis* infections are also the most prevalent bacterial infection worldwide, with roughly one third of the world's population latently infected with *M. tuberculosis* (34). During infections with *M. tuberculosis*, bacteria invade alveolar macrophages and are sequestered into granuloma structures as other immune cells infiltrate the area to contain the infection with the infected macrophages at the center of the granuloma. Within these infections there is frequently a subset of persister bacteria. *M. tuberculosis* replicates very slowly, (even under ideal laboratory conditions), which may also add to the high degree of persistence seen in these infections (19, 42).

Persistence within a *M. tuberculosis* infection is caused by several factors. Slowed growth and metabolism, which are two of the most well-supported causes of antibiotic persistence, greatly limit the effectiveness of antibiotic treatment in *M. tuberculosis* infections. The subpopulations of persistent bacteria within a *M. tuberculosis* infection greatly contribute to the length of treatment required to eradicate *M. tuberculosis* from patients. Antibiotic treatment can last nine months and must be treated with a combination of antibiotics including rifampin, isoniazid, and fluoroquinolones. Long courses of antibiotics, such as those required for the treatment of *M. tuberculosis*, increase the total volume of antibiotics in the environment (33). This is another avenue by which persistent bacteria can indirectly contribute to antibiotic resistance, by increasing the total amount of antibiotic required to treat infections. Prolonged exposure to antibiotics during treatment, and failure of patients to comply with a long and

expensive treatment, can also lead to resistance within the population of the bacterial infection, which has led to the emergence of MDR-TB and XDR-TB.

Other common bacterial pathogens, such as *S. typhi* and *S. aureus*, spread systemically to establish infection in deep tissues such as the liver and spleen, and are additional examples of common, persistent bacterial infections (9, 39). Deep tissue infections can arise after systemic infection, when bacteria spread from one tissue into the bloodstream, and then seed other tissues. These infections, like all other tolerant or persistent infections, can then lead to recurrent infections after completion of treatment for a systemic infection because bacteria may remain within deep tissues (43). In deep tissue sites, antibiotics may not accumulate to levels required to completely clear the infection. In some cases, interactions with the host or other bacteria in deep tissue sites can lead to the development of a persistent population of bacteria in the infection, which further complicates treatment. For example *S. typhi* has been shown to enter a persistent state after macrophage engulfment while growing within an intracellular salmonella-containing vacuole (44).

Repeated treatments for recurrent infections negatively impact patients who suffer from these infections, both physically and financially, and can lead to more severe infections if left untreated (45). Uropathogenic *E. coli* (UPEC) is one of the most well-studied bacteria that establishes persistent infections. The mechanisms of persistence used by UPEC, discussed below, are significantly different from those that lead to persistence in deep tissue and *Salmonella* infections (36). These infections, unlike deep tissue infections, do not arise from systemic infections, but rather from local infections leading to urinary tract infections (46). Studying this system can shed light on treatment for urinary tract infections that are one of the most common bacterial infections in women. Individuals with recurrent urinary tract infections

require multiple, frequent rounds of antibiotic treatment and, if left untreated, these infection can lead to more dangerous kidney infections (46).

Causes of Persistence

While there are many well-established mechanisms of antibiotic resistance, the causes of persistence remain less clear and need to be further elucidated in order to develop therapeutics that directly target persistent bacteria. Antibiotic persistence has been more difficult to study because the conditions that cause persistence can be difficult to recreate *in vitro*. Studying antibiotic persistence is further complicated because persistence may not be specific to a single antibiotic. Dormancy or slowed metabolism within a population can lead to multidrug persistence. Dormancy or quiescence is a state of non-division that can be entered by many environmental bacteria and *M. tuberculosis*. Dormancy allows bacteria to survive in inhospitable environments such as starvation conditions. Because of this broad protective effect provided by dormancy, it can be triggered by multiple redundant pathways (47). There are also multiple stress response pathways that can slow growth and metabolism while not entirely arresting growth, leading to persistence during infections. These pathways can be dependent on both host and bacterial factors and are initiated by multiple different environmental stressors. Recent advances, such as RNA sequencing and other genomic and proteomic approaches, have allowed researchers to learn more about these pathways.

The events that induce persistence are varied and range from exposure to reactive oxygen species (ROS) and signals from other bacteria within the environment, to direct responses to the antibiotic itself (Figure 2) (15, 16, 48, 49). Some common pathways that lead to persistence are the SOS response along with the secondary signal tetraphosphate or pentaphosphate guanosine (pppGpp or ppGpp), activation of toxin-antitoxin systems, and

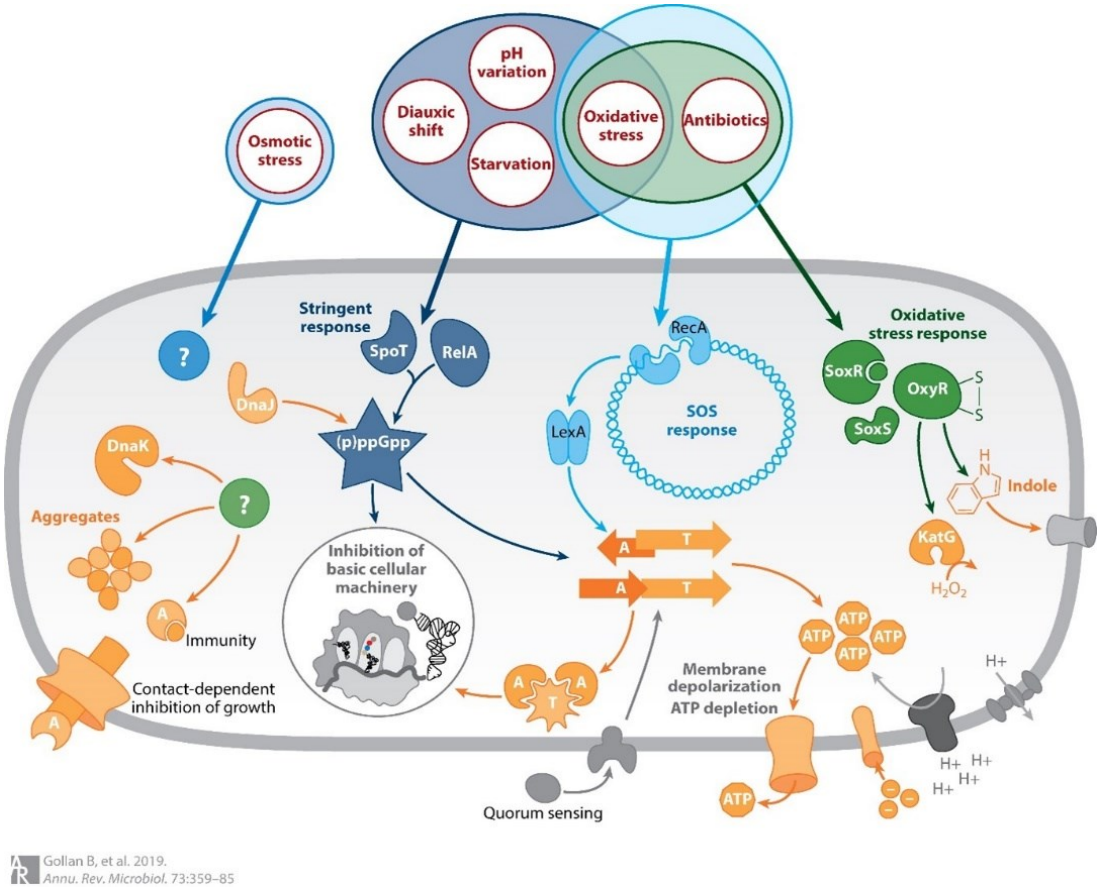


Figure 2. Extracellular signals that cause antibiotic persistence. (Adapted from Gollan et al., 2019) Extracellular signal of antibiotic persistence are circled in red. Known stress response pathways triggered by these signals are in blue and green and known effectors of these pathways are shown in orange.

responses to oxidative stress (17, 26, 40). Many of these pathways can lead to reduced metabolism, which is currently thought to be the strongest driver of persistence (37). However, slowed metabolism is not the only cause of persistence. Other mechanisms include inducing efflux pump expression or the expression of an antioxidative response (19, 50). Antioxidative proteins can prevent killing through the accumulation of ROS which is thought to be key way that antibiotics cause bacterial cell death. It is important to note that there is a set rate of persister formation even in ideal conditions and there is always a subpopulation of persistent cells in the population. The subpopulation of persisters can expand when the entire population experiences stressors. Stressors such as limited access to nutrients and interactions with the immune system within the host environment can induce the SOS response or toxin-antitoxin systems and induce persistence (38, 51).

Nutrient Limitation

It has recently been demonstrated that when uncoupled with growth, metabolism is a better indicator of antibiotic susceptibility to bactericidal antibiotics (37). This may have to do with the reduced availability of antibiotic targets during slowed growth. Many antibiotics require cellular activity for efficacy. β-lactams that target the cell wall, for example, require active cell wall synthesis to kill bacteria. Other antibiotics that target protein synthesis, such as tetracyclines that bind the 30S ribosomal subunit, may not be effective if the bacteria are dormant and not translating protein.

Starvation, or nutrient limitation, was one of the earliest described inducers of persistence in bacterial infections, and slowed metabolic rates remain one of the most well-studied inducers of persistence (37). While many factors can lead to persister cell formation as a result of starvation, it is most often linked to the SOS response and (p)ppGpp accumulation. In *E.*

coli the SOS response is initiated by RecA being recruited to single-stranded DNA. This leads to the auto-proteolysis of LexA which allows for the expression of genes that are repressed by LexA. The SOS response has global and varying effects on cells including dormancy caused by activation of the TisB toxin-antitoxin module. Starvation can also induce an SOS response in which bacteria produce (p)ppGpp signaling a transcriptional switch to a dormant phenotype (38). Similarly, when bacteria reach stationary phase, the point in growth when division stops, they become much more tolerant to antibiotics. This is also due in part to slowed growth and an accumulation of (p)ppGpp (52).

The accumulation of (p)ppGpp in bacterial cells and the SOS response can also promote biofilm formation, which can impact antibiotic efficacy. Biofilms are well known to cause persistent infections associated with medically indwelling devices, and biofilms can promote severe infections in cystic fibrosis patients who are colonized with *Pseudomonas aeruginosa* (15). While it was long thought that persistence in biofilms was due to an inability of antibiotics to reach a high concentration in the center of the biofilm colony structure, it has recently been demonstrated that some antibiotics can penetrate biofilms (18, 53). This indicates that the high level of persisters that are present in biofilms are probably playing a more significant role in the recalcitrance of biofilms, compared to the ability of antibiotics to accumulate within biofilms (18). Nutritional stress can occur during infection when a population of bacteria has either used the local nutrients that are required for growth or as a result of sequestration of nutrients such as iron. Interactions with the host immune system can also arrest growth.

Toxin-Antitoxin Systems

Toxin-antitoxin (TA) pathways are another major group of signaling pathways that can induce persistence through arresting growth. TA systems encode a toxin that is harmful to the

bacteria along with the antitoxin that prevents its activity. In ideal conditions the ratio of toxin to antioxin is 1:1 to prevent toxin activity. When the balance of toxin to antitoxin favors the toxin, the toxin is no longer effectively neutralized and can affect it's cellular targets. Toxins can have a wide range of effects include inducing biofilm formation, disrupting proton motive force, and inhibiting transcription and translation (24). Proton motive force is the proton gradient established in the bacterial cell membrane that is used for ATP synthesis (54).

There are two types of TA systems, type I and type II, that have been studied in the context of persistence. Type I TA antitoxins are antisense RNAs that prevent expression of their conjugate toxin by binding mRNA transcripts and inhibiting translation (10). Type I toxins are generally small pore forming toxins that insert into the bacterial membrane and disrupt ATP synthesis by altering the proton motive force (10). The ability of type I TA modules to induce persistence has been well characterized in the K12 lab strain of *E. coli.* Type II antitoxins are proteins that directly act on their toxin protein to inactive them. The functions of these proteins are much more diverse; however, many inhibit translation by acting on mRNAs, tRNAs or even rRNAs.

Type I and II TAs can also be activated by the SOS response and in response to (p)ppGpp accumulation. The activity of these systems may not completely inhibit bacterial growth, but it can drastically reduce the growth rate and metabolic activity of bacterial cells, and lead to an increased number of persister cells within the population. The study of these pathways, like the study of the other causes of persister formation, is complicated by the considerable redundancy that has been observed in these pathways in *E*. *coli.*

Induction of persistence and chronic infection by TA modules have also been studied in *S. aureus.* The MazEF TA module in *S. aureus* has been implicated in regulating biofilm

production and antibiotic persistence *in vitro* and in a murine model of infection (24). The toxin MazF is an endoribonuclease that cleaves single stranded ACA sequences in mRNA to inhibit translation (24). MazF has been shown to drive tolerance in biofilms in many Gram-negative bacteria, and as expected MazF deficient mutants show increased susceptibility to antibiotics within biofilm communities. It was also demonstrated that loss of the *mazF* toxin leads to increased biofilm production. This was not an expected result. Further investigation revealed that biofilm formation was dependent on the *ica* operon and the expression of the polysaccharide intracellular adhesin (PIA). PIA helps in regulation of biofilm production and increased levels of the adhesin in the absence of MazF indicate a regulatory role of MazF in the expression of PIA. This result is interesting as biofilm production is often corelated with antibiotic persistence and this demonstrates that he biofilm in isolation may not be completely protective (24).

Immune Interactions

The host immune system is thought to induce persistence in multiple ways. The innate immune response initially controls many bacterial infections through interactions with neutrophils or macrophages. These innate cells utilize mechanisms such as phagocytosis and the production of reactive oxygen species (ROS) and secreted reactive nitrogen species (RNS) to contain and eliminate bacteria (23, 50). The same responses that are effective at controlling the spread of bacteria also inhibit growth or stress bacterial cells and can lead to antibiotic persistence (50). One area with a significant body of research in this area is the role of ROS in the induction of persistence. There are several well-described pathways down stream of ROS exposure that can lead to tolerance such as the induction of the SOS response and the mobilization of an antioxidative response to mitigate antibiotic killing through the production of ROS. Stressors experienced in the phagosome following phagocytosis, which include exposure to

ROS among conditions such as a low pH, have also been shown to induce persistence. Other immune interactions such as exposure to RNS have been shown to induce tolerance in some systems, however this area remains poorly understood.

There are several proposed mechanisms through which interactions with RNS and ROS lead to persistence, and these mechanisms may be species and antibiotic specific. In some species such as *Streptococcus mutans,* it appears interaction with host-derived ROS promotes the expression of antioxidant defenses and prevents killing by antibiotics that kill through the production of ROS (50). This, however, does not hold true for other systems, in which it appears that corruption of central metabolism or breakdown of proton motive force increases the rate of metabolically inactive persisters (17). A third, less common, mechanism by which immune cells can cause tolerance is the induction of efflux pumps. The induction of efflux pumps is not dependent on the presence of ROS or RNS. The engulfment into macrophages promotes the expression of the Rv1258c efflux pump in *M. tuberculosis*., which confers rifampin tolerance. This interaction has been shown in multiple *M. tuberculosis* group clinical isolates (19).

Oxidative and Nitrosative Stress

The role of ROS in antibiotic killing of bacteria is currently a debated field because oxidative stress can induce persistence as well as lead to bacterial cell death. ROS have been shown to induce persistence in *M. tuberculosis*, *E. coli, S. mutans,* and *S. aureus* (50, 55). The induction of persistence through ROS stress is of particular importance during infection. Macrophages eliminate bacteria after phagocytosis with oxidative bursts. Bacteria that are able to survive this interaction may become persistent.

S. mutans is a bacterium that is part of the normal oral microflora. This bacterium can cause biofilm formation in the mouth, leading to cavities, as well as cause infective endocarditis

and bacteriemia when it is introduced into the bloodstream. Oral bacteria can be introduced into the bloodstream during dental surgery and even while brushing teeth. During extra-oral infections, *S. mutans* will also form biofilms, and can be difficult to treat due to antibiotic persistence that is commonly observed in biofilm communities. Persistence in *S. mutans* biofilms has been directly linked to oxidative stress. When the transcriptional regulator SpxA1, which regulates the expression of genes related to oxidative stress, was knocked out, *S. mutans,* bacteria within biofilms were significantly more susceptible to gentamicin, vancomycin, and linezolid. This demonstrates that the ability to rapidly respond to oxidative stress can have a protective effect against many bactericidal antibiotics, which may cause death through generating oxidative stress (50). ROS produced by the immune system can also lead to persistence by altering bacterial metabolism (17).

The impact of altered metabolism caused by ROS on the induction of persistence has been studied in *S. aureus. S. aureus* is a common pathogenic bacterium that causes a wide array of infections in humans. Many of the more serious infections, such as sepsis, are a result of bloodstream infections that can be initiated by skin infections. When bloodstream access results in colonization of blood-rich organs, these infections are referred to as deep tissue infections. Deep tissue infections can be difficult to clear with antibiotic treatment, even in strains of *S. aureus* that are susceptible to antibiotics due to their limited diffusion in deep tissues. *S. aureus* infections are primarily controlled through phagocytosis by neutrophils and macrophages; however, phagocytosis by macrophages can lead to persistence though the generation of ROS. *S. aureus* can also avoid killing by macrophages, which can lead to the establishment of a reservoir for reinfection within macrophages. Macrophages kill phagocytosed bacteria through the oxidative burst. It has recently been demonstrated that the oxidative burst, while killing the majority of bacteria, can also lead to multidrug persistence in the surviving bacteria. This was

shown by infecting macrophages that were activated by exposure to LPS and IFNγ with *S. aureus*, which led to intracellular ROS exposure. These macrophages were then treated with rifampin to determine if the intracellular bacteria were killed. It was proposed that the inactivation of the enzymes aconitase and succinate dehydrogenase in *S. aureus*, caused by interactions between ROS and Fe-S clusters in the enzymes, led to disruptions in the central metabolism of *S. aureus* and persistence (55). Because ROS are an important component in the immune response to bacterial infections, it is difficult to develop and use drugs that inhibit these pathways and target persistent bacteria. Recovering metabolic pathways inhibited by ROS or targeting enzymes that inhibit killing by ROS present more attractive future antimicrobial compound targets.

In addition to ROS, the host immune system can also induce persistence in other ways. IFNγ-primed macrophages have also been shown to induce persistence to beta-lactams through the production of RNS in *Burkholderia pseudomallei* and nontyphoidal *Salmonella.* In this system, it was shown that the collapse of PMF and inhibition of central metabolism plays a stronger role than mounting an antioxidative defense in the development of persistence in this system. This may indicate that the persistence observed here is a product of inducing persisters by interfering with metabolism in these populations (23).

RNS have also been found to promote persistence in a *Y. pseudotuberculosis* model of infection. During an infection with *Y. pseudotuberculosis,* nitrosative stress from macrophages and monocytes is thought to be important in controlling the early stages of infection. As with many pathogenic bacteria, *Y. pseudotuberculosis* expresses nitric-oxide-reducing enzymes in response to stress from RNS to protect the bacterial population from these antimicrobials. Heterogeneous expression of the nitric-oxide-reducing gene, *hmp,* has previously been described in *Y. pseudotuberculosis* spleen infections, where a subpopulation of bacteria at the

periphery of pyogranulomas responds to the RNS-derived stress and produces *hmp.* These *hmp*-expressing bacteria preferentially survive treatment with doxycycline, implying that the nitrosative stress is inducing antibiotic persistence in the exposed population. Only about half of the surviving bacteria are from the *hmp*-expressing population however, indicating that there may be other factors contributing to persistence in this system. (7)

Internalization of *Salmonella typhimurium* by macrophages has also been shown to induce persistence to cefotaxime, a 3rd generation cephalosporin, *in vitro*. Interestingly, the persistent non-dividing subpopulation maintains SPI-2 type-III secretion system activity to avoid killing within the macrophage. Cephalosporins target cell wall synthesis so in this model growth of the cells is necessary for the antibiotics to kill bacteria, so it may not hold true with other classes of antibiotics. (44)

Antibiotic Stress

Antibiotic stress can lead to persistence by inducing dormancy. The stress from daptomycin has been shown to induce multidrug persistence in *S. aureus* (8). Bactericidal antibiotics such as ciprofloxacin were used to induce a persistent phenotype that was not metabolically active in experiments to establish that activity of the SPI-2 type three secretion system is necessary for persistent *S. typhimurium* to survive in macrophages. *S. typhimurium* treated with ciprofloxacin were persistent with treated with other antibiotic however they were eliminated by activated macrophages (44).

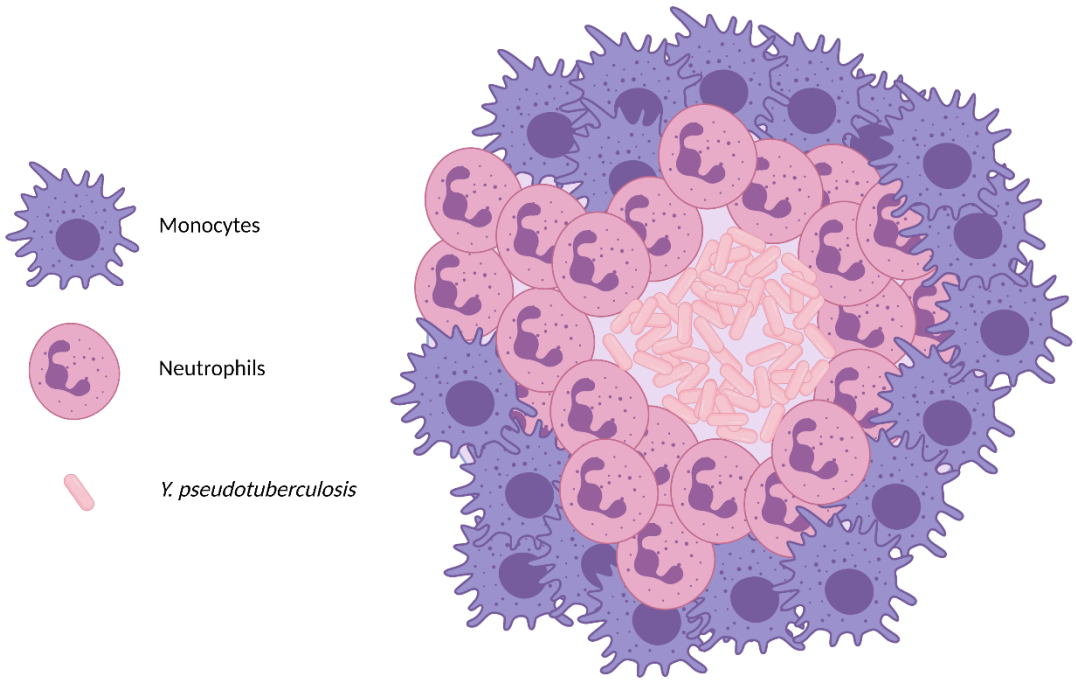
Antibiotic stress can also lead to persistence by promoting invasion of host cells. Intracellular bacteria are generally more protected from antibiotics than extracellular bacteria because many common antibiotics fail to adequately penetrate human cells and reach inhibitory levels. UPEC is known to establish biofilm-like communities in the bladder epithelial cells. It is

thought that these are a contributing factor to recurrent UTIs as well as persistence that is commonly observed in UPEC. Sub-lethal doses of antibiotics can drive normally extracellular bacteria *Bacillus cereus* and *S. aureus* to invade host cells by inducing toxin expression that promotes invasion. *B. cereus* is not a major cause of human disease; however, it is known to cause food poisoning. Low doses of antibiotics were shown to induce non-hemolytic enterotoxin (Nhe) production in *B. cereus* and α-toxin expression in *S. aureus*. The production of toxins by each of these species was also shown to promote invasion into IEC-6 cells, an intestinal epithelium cell line. In this model the antibiotics play a dual role in both prompting invasion and inhibiting essential cellular processes such as autophagy, to prevent the clearance of intracellular bacteria (56).

Inhibitory doses of antibiotics have also been shown to enrich persister formation after high-dose, extended-interval therapy that is required when using more toxic antibiotics to clear infections that are resistant to other options. This method of therapy is common when treating the nosocomial "ESKAPE" bugs (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter spp.),* which are often multidrug resistant. After treating this group of bacteria with repeat high doses of aminoglycosides there was between a 37- and 213-fold increase in the formation of persister cells from evolved clones relative to the pretreatment rates of persister formation. (16)

Yersinia pseudotuberculosis Model

Yersinia pseudotuberculosis is a gram-negative enteric pathogen that can infect the intestinal epithelium and cause disseminated infection by accessing the lymphatic system. It is also the most recent common ancestor of *Y. pestis,* the causative agent of bubonic, septicemic and pneumonic plague. In an intestinal infection, which is its natural route of infection, *Y. pseudotuberculosis* invades M cells within the intestinal epithelium and utilizes these to cross



Created with BioRender.com

Figure 3. *Y. pseudotuberculosis* microcolony model. *Y. pseudotuberculosis* forms microcolonies surrounded by immune cells. In direct contact with *Y. pseudotuberculosis* are neutrophils that are in an inactive state due to the activity of the *Y. pseudotuberculosis* type III secretion system. Directly surrounding the layer of neutrophils is a layer of monocytes.

the intestinal barrier. *Y. pseudotuberculosis* can then colonize Peyer's patches (aggregates of lymphatic tissue in the intestinal ileum that survey the intestinal microbiota) and mesenteric lymph nodes. Based on studies in mouse models of infection, subsets of bacteria remain within the intestinal lumen, and have been shown to directly access the bloodstream to cause systemic, disseminated infection (57). During systemic infection, *Y. pseudotuberculosis* will infect blood-rich organs, such as the liver and spleen, causing infection of these deep tissues. In deep tissue infections, *Y. pseudotuberculosis* establishes clonal, extracellular microcolonies that allow for the study of heterogeneous gene expression, and specifically expression of genes that promote infection, or virulence factors (58).

Like all other pathogenic *Yersinia* species, *Y. pseudotuberculosis* has several mechanisms to evade the host immune response, such as expression of a type III secretion system (T3SS) that injects Yop (Yersinia outer proteins) effectors into host immune cells. *Y. pseudotuberculosis* typically utilizes the T3SS to target neutrophils and prevent activation and phagocytosis (57). Neutrophils and monocytes play an essential role in the containment of *Y. pseudotuberculosis* infections at early stages, before the infected individual succumbs to the infection. In immunocompromised individuals that cannot adequately contain the infection in the intestinal epithelium *Y. pseudotuberculosis* will cause disseminated infections and establish colonies in lymphatic tissues. The mouse model of infection more accurately recapitulates infection in immunocompromised individuals as *Y. pseudotuberculosis* can escape the intestine and mice will succumb to the infection.

In tissue infections, *Yersinia* forms microcolonies or pyogranulomas with a distinct architecture protected by a layer of neutrophiles that are unable to carry out phagocytoses due to the activity of the T3SS (Figure. 3). Surrounding the layer or neutrophils is a population of monocytes that are not in contact with the bacteria but are able to interact with the bacteria

through secreted factors such as RNS that diffuse into the microcolony (58). In response to these stressors, *Y. pseudotuberculosis* forms distinct bacterial subpopulations responding to the different types and amounts of host-derived stress. The structure of the microcolonies also effects diffusion of antibiotics such as doxycycline into the microcolony. It has been demonstrated by our lab that doxycycline accumulates to different levels in the periphery and the center of the microcolonies, accumulating to between 100ng/ml and 1µg/ml in the periphery and around 10ng/ml in the centroid. The diverse stressors experienced by different bacteria and clonal nature of these microcolonies makes them a useful tool to study the multiple causes of persistence, including host-derived stressors and exposure to antibiotics (7).

Mouse Model of Y. pseudotuberculosis Infection

To study the effects of these stressors *in vivo,* our laboratory utilizes a mouse model of infection. Several routes of infection are used in mouse models to study host-microbe interactions in the deep tissue stage of *Y. pseudotuberculosis* infection: an intravenous infection, infection through feeding, intraperitoneal injection (IP) and oral gavage. Each route of infection offers advantages and disadvantages. The intravenous route of infection utilized in this thesis allows for a more controlled load of bacteria to be administered, in contrast with other models commonly used to study *Yersinia* infections *in vivo*. Directly introducing the bacterium into the bloodstream also ensures a consistent time frame for the infection and ensures that the infection will spread systemically and establish an infection in lymphoid organs such as the spleen. Oral infections, through either gavage or feeding, may not deliver a consistent inoculum to the spleen or other organs to set up a deep tissue infection. When mice are orally infected with *Y. pseudotuberculosis,* they pass bacteria in their stool and can become reinfected, or ingest a higher number of bacteria, than the experiment intended. In addition, gavage can cause damage to the throat which may affect disease progression. The oral routes of infection more

closely mimic natural routes of infection. Due to the higher degree of variability with these models, however, we chose to use an intravenous route of infection. In an intraperitoneal (IP) infection model, it is not clear where or how the bacteria access the interior of organs and the bacterial load is less controlled. Tail vein infection more closely approximates the downstream bloodstream access that occurs during natural infection than IP and allows for a more controlled number of bacteria to be delivered directly to the spleen. *Y. pseudotuberculosis* is an excellent model to study the effects of antibiotics *in vivo* because of the genetic tools that are available to modify it and the ease with which its genome is modified. In addition, the genome has been annotated, making genomic work more accessible.

Adaptation Response to Doxycycline

Doxycycline is commonly used to treat *Yersinia* infections in humans. In the mouse model of infection, *Y. pseudotuberculosis* is able to survive one treatment with an inhibitory dose of doxycycline. It is not known what allows *Y. pseudotuberculosis* to survive treatment. The diverse stressors that are experienced by *Y. pseudotuberculosis* may lead to persistence that allow for the observed survival. The concentrations to which doxycycline accumulate within a microcolony may lead to adaptations by the bacteria that allow them to survive antibiotic treatment.

To determine if there is an adaptation in response to doxycycline, RNA-seq was performed on populations of *Y. pseudotuberculosis* that had been exposed to a concentration of 100ng/mL of doxycycline. Samples were taken at 2 hours and 4 hours for RNA isolation (Figure 4.). From this experiment four genes with at least a two-fold change and significantly differential expression when compared to an untreated sample were identified at the two hour time point only. By the fourth hour the difference was no longer significant. Two of these genes, *osmB* and *ompF,* showed increased expression in response to doxycycline, and two genes, *tusB and CNFy,*

*s*howed reduced expression. It was then necessary to determine if the altered transcription levels of these genes effect antibiotic susceptibility of *Y. pseudotuberculosis* and, potentially, how the gene products are providing protection.

Experimental Approach

To determine if the adaptation to subinhibitory doses of antibiotics can promote antibiotic persistence during *Y. pseudotuberculosis* infection, deletion strains for the upregulated genes *osmB* and *ompF* were tested to measure their antibiotic susceptibility and accumulation. OmpF is an outer membrane porin that is upregulated in response to osmotic stress (21). In many studies with *E. coli,* porin-deficient mutants have been found to be more tolerant than wild type bacteria, so it is particularly interesting that increased expression of this protein was associated with exposure to antibiotics (59). OsmB is an outer membrane lipoprotein about which little is known. It has been observed that the presence of OsmB in the outer membrane allows for rapid remodeling of the outer membrane following osmotic stress, but it is unclear how this would aid in response to a bacteriostatic antibiotic such as doxycycline (20). Based on the location of both of these proteins in the outer membrane, we hypothesized that they may be affecting the accumulation of antibiotics within the cell. This may be mediated by allowing passive diffusion out of the cell through OmpF or by OsmB altering the composition of the membrane to inhibit diffusion into the cell. We expected to observe higher susceptibility in the knockout strains if these genes played an important role in the development of persistence. Accumulation of antibiotics was measured within individual and dual knockouts to determine if there was a difference in accumulation of antibiotics we predicted in these mutants.

Methods

Bacterial Strains and Culture Conditions

The mutant *ΔosmB, ΔompF*, and *ΔosmB ΔompF* strains of *Y. pseudotuberculosis* were derived from the IP2666 strain a clinical isolate first identified in France (60, 61). Overlap extension PCR was used to generate a fragment with 1kb up- and downstream regions fused together, lacking the gene of interest. This was ligated into a suicide vector, introduced into IP2666 by conjugation, and homologous recombination was used to incorporate the deletion construct into the chromosome. Insertion of the deletion construct was confirmed by PCR(62). IP2666 and all derived strains were grown in Miller's Luria-Bertani (LB) medium, on LB agar, or in 2xYT broth for plasmid purifications and infections. 2xYT broth contains twice the yeast extract and tryptone as Miller's LB. To maintain reporters on plasmids the media and agar plates were supplemented with 25μg/ml of chloramphenicol (Cm), 100μg/ml of carbenicillin (Carb), or both antibiotics in some cases. Broth cultures and LB agar plates of all strains were grown at 26°C, to avoid the virulence factor expression in *Y. pseudotuberculosis*. Growth at 37°C induces expression of many virulence factors in *Y. pseudotuberculosis* (63-65). *Y. pseudotuberculosis* cultured LB agar plates were grown for 2 days. Broth cultures were grown with rotation to ensure aeration of the culture and to promote robust growth. For *in vitro* experiments all strains were grown at 37°C to induce virulence factor expression and more accurately model conditions experienced in infection.

Plasmid Preparation and Purification

Previously described reporters for tetracycline were used to construct reporter strains for *ΔosmB, ΔompF,* and *ΔosmB ΔompF* mutants. To isolate fresh plasmids for the transformations the Promega PureYield Plasmid Midiprep System (Promega Corporation, Madison, WI, USA, cat no A2492) was used. To perform the plasmid purification 50ml 2xYT

broth cultures of the *E. coli* strain containing the plasmid of interest are grown overnight at 37°C with shaking. Following the overnight growth, the cultures must be transferred to a sterile 50 conical tube and pelleted at 5000xg for 10m. Following the pelleting step, the cells are resuspended in 3ml of resuspension solution using a serological pipette. When the cells are completely resuspended 3ml of lysis solution were added to the mixture. The 50ml conical tubes were rotated by hand to mix the lysis solution until the mixture became snotty. Upon complete lysis of the cells, 5ml of neutralization solution were added to the mixture and inverted to mix. The addition of the neutralization solution should cause the formation of a white precipitate. The lysate was then centrifuged at 4000 rpm for 15 m to pellet the precipitate out of solution. The supernatant was then carefully poured into the clearing column included with the kit stacked on top of the binding column in a vacuum manifold and the vacuum was applied to pass the supernatant through both columns. Following the initial use of the clearing column it was removed. To remove any LPS contaminants, 5ml of endotoxin removal solution was added to the column and pulled through with the vacuum. A final wash of 20ml of column wash solution was added and pulled through the column with the vacuum before the plasmid was eluted in 600µl of dH_2O.

Transformation of *Y. pseudotuberculosis* to Introduce the Tetracycline Reporter

To measure antibiotic accumulation within the *ΔosmB, ΔompF,* and *ΔosmB ΔompF* strains, a reporter based on the tetracycline resistance operon (Tre) was introduced into the deletions by transformation using electroporation (66). To transform *Y. pseudotuberculosis*, the strain of interest must first be grown overnight for 16 to 18 hours in LB broth with the appropriate antibiotics. After 16 to 18 hours growth the broth culture is diluted 1:50 into fresh LB and grown for two and a half to three hours to move the cells into the log phase of growth for electroporation. Following the incubation period, 1mL of cells from the dilution are pelleted

for 1m at 15,000rpms at 4°C in a sterile microcentrifuge tube. The pelleted cells are put immediately on ice after removal from the microcentrifuge and the supernatant is then pipetted off. The cells are then resuspended in 1mL of ice-cold dH_2O. The pelleting and resuspension steps are repeated 2 more times. The cells are then pelleted once more using the same conditions and resuspended in 50µL of ice-cold dH_2O. 2µl of the plasmid pMMB67EH containing the P_{tetA}*::mCherry* reporter at a concentration of roughly 150ng/µl were added to the resuspended cells on ice. The plasmid cell mixture was then added to a pre-chilled 2mm cuvette for electroporation. The cells were electroporated using a pulse at 2.2 kV for 4.6 to 4.8ms. Immediately following electroporation, 950µl of LB are added to the cuvette. The cells are then transferred to a sterile microcentrifuge and allowed to recover for 2 hours at 26°C. Following the 2-hour recovery, the cell mixture was pelleted and resuspended in 100µl of LB to be plated on an LB plate containing Carb to select for transformants containing the plasmid (pMMB67EH). To confirm that the transformants contained the plasmid, a colony PCR was run on 8 colonies for each of *ΔosmB, ΔompF,* and *ΔosmB ΔompF* strains using the primers 5'GTGTCGCTCAAGGCGCACTC3' for the pMMB67EH backbone and 5'GTCGTCGACTTAAGACCCACTTTCACATTTAAGT3' for the *tetR* gene contained in the Tet reporter. The same protocol was followed for the transformation of the plasmid pACYC184 containing a constitutive *gfp* into the P_{tetA}*::mCherry*-containing *ΔosmB, ΔompF,* and *ΔosmB ΔompF* strains. After recovery from electroporation, the cells were plated on LB containing Carb and Cm to select for transformants containing both plasmids. Of the transformants, eight were screened for each of the three strains using the primer 5'CACAGTTAAATTGCTAACGCAGTCAGGCAC3' for the pACYC184 vector backbone and 5'GTCGTCGACATGAGTAAAGGAGAAGAA3' primer for the gfp insert .

Colony PCR

To confirm by PCR that the strains had up taken the plasmid and that the gene of interest was maintained in the plasmid, colony PCR was performed on eight colonies from each transformation (63). A colony PCR can be performed from a whole bacterial colony. To carry out the PCR, 2X OneTaq mastermix (MM) was used. First a master mix of the 10 µM primers, and OneTaq MM was made. All bacterial colonies were resuspended and lysed in 100µl of dH_2O in sterile microcentrifuge tubes. 13.5µl of master mix was added to each test tube along with 11.5µl of dissolved bacteria. The PCR was run with a 94°C initial denaturation followed by 30 cycles of a 30s 94°C denaturation, a 30s annealing at 60°C, and 68°C extension for 90s. Following the 30 amplification cycles, there was a final extension for 5m at 68°C. The products were run out on a 1.5% agarose gel alongside a 2 log DNA ladder to confirm the expected DNA fragment was present and it was the correct size.

Competitive Survival

Overnight of a *Y. pseudotuberculosis* IP2666 containing a constitutive *gfp* in a pACYC184 backbone (Cm resistant) and the Δ*osmB* strain (Cm sensitive) were grown separately in 2ml LB broth cultures at 26°C with rotation for 16-18 hours. The cultures were then diluted 1:100 separately into fresh 2ml LB broth cultures and incubated for 2 hours at 26°C with rotation to re-enter log phase of growth. After the 2-hour incubation, the strains were mixed in equal volumes and diluted 1:1,000 into 300µl of LB in 96-well plate and treated with 100ng/ml, 1µg/ml, 4µg/ml, or left untreated. Single strain controls were also set up following the same process, using the same treatments. The 96-well plate with the cultures was then incubated for 4 hours at 37°C, with time points taken at 0 hours, 2 hours, and 4 hours to be plated on LB or LB Cm agar plates. At each time point 80µl of culture for each condition was removed and put on ice in a fresh 96-well plate. To obtain accurate CFU counts a serial dilution to a 10^{-3} dilution was

performed on the sample. Following the serial dilution, three 10µl spots of each dilution for each treatment condition were plated for the mixed sample containing the Δ*osmB* strain and the *gfp+* on both LB and LB CM agar plates. The Δ*osmB* single strain control was collected and diluted in the same manner and plated on LB, and the *gfp+ Y. pseudotuberculosis* was plated LB following the same plating and dilution method. All plates were grown for 2 days at 26°C before counting. Bacterial growth on LB plates depicts the total bacterial count, while colonies on LB/Cm are specifically the number of wild type (WT) cells within the cultures. The number of mutant cells is calculated by subtracting the number of WT cells from the total. After the colonies were counted, a competitive index (CI) was established for the mixed sample by subtracting the average colonies on the LB CM plates from the LB plates for each condition to establish the CFUs for the mutant. To establish a competitive index the formula (mutant/WT timepoint)/(mutant/WT inoculum) was used.

Viability Assay

To measure the single strain susceptibility of the Δ*osmB* compared to wild type IP2666 a single strain viability assay was performed to determine the number of cells that survived treatment with doxycycline. This was done by first growing broth cultures of Δ*osmB* and IP2666 at 26°C for 16-18 h. These cultures were then diluted 1:100 into 2ml of LB broth and incubated for 2 hours at 26°C to reenter log phase. Following the 2-hour incubation the cultures were diluted 1:10,000 into fresh LB broth and treated with doxycycline at 100 ng/mL, 1 µg/mL, 4 µg/mL or left untreated. The new cultures were then incubated at 37°C for 4 hours. Samples were taken at 0 hours, 2 hours, and 4 hours. To measure the number of viable bacteria in the sample the CUFs were counted by first diluting the sample to 10^{-3}. Following the dilution three 10µl spots of each dilution of each treatment condition was plated for each sample on LB plates. All plates were grown for 2 days before counting.

In vivo Antibiotic Permeability

To measure the effect of the mutations on the accumulation of doxycycline *in vivo,* *o*vernight cultures of bacterial strains IP2666, *ΔosmB, ΔompF,* and *ΔosmB ΔompF* containing the tetracycline reporter and a constitutive *gfp* were used to infect five 6- to 8-week-old female C57BL/6 mice through tail vein injections. Overnights of each strain were grown in 2ml of 2xyT broth at 26°C for 16 to 18 hours with rotation in plastic test tubes. The cultures were then diluted 1:10,000 into filter sterilized sterile PBS to prepare an inoculum with roughly $1x10^3$ CFU of *Y. pseudotuberculosis* in 100µl. To perform tail vein injections, mice were warmed under a heat lamp to cause the vein to dilate until they showed signs of being warm, such as washing their face and slowed movement. Once the mice were sufficiently warmed, a mouse is taken from the cage and placed in a brass restrainer. Once the mouse was restrained the tail was sprayed with 70% ethanol to both ensure the injection was not introducing bacteria from the tail into the blood and to cause the vein to come closer to the surface. The vein was injected with 100µl of the inoculum and placed in a clean cage. Following the infection, the inoculum is serial diluted to 10^{-3} and three 10µl drops were plated for each dilution to confirm the 10^3 CFU/100µl dose of the inoculum. The mice were treated with 100µl of 7.2mg/ml of doxycycline 48 hours after infection via intraperitoneal injection. 24 hours following the treatment the spleens of the mice were harvested. Half of each spleen was homogenized, serial diluted to 10^{-3}, and plated to determine CFUs per spleen. The other half of the spleen was fixed in 4% PFA in PBS overnight at 4°C, frozen-embedded in OCT, and stored at -80°C to be sectioned and imaged.

Staining and Imaging of Embedded Spleens

Spleens embedded in OCT were sectioned into 10µm sections. The sections on slides were thawed in PBS for 20 m, then stained with 100µl of a 1:10,000 dilution of Hoechst. The stained sections were then washed for 2 m in PBS 3 times and mounted with Prolong Gold

antifade mounting media and a glass coverslip. The mounted slides were allowed to cure overnight in a drawer to prevent bleaching. Sections were scanned at 200x magnification to identify any microcolonies, images of microcolonies were then captured at 630x using three fluorescent filters DAPI (excitation 358nm, emission 460nm), eGFP (excitation 488 nm, emission 510 nm), and mCherry (excitation 580nm, emission 610nm). Imaging was done using an apotome to deconvolute background in the images. This process was repeated for all microcolonies in all the collected tissues.

In vitro Antibiotic Diffusion

To test the effects of the genes of interest on antibiotic accumulation in bacterial cells *in vitro* 2ml LB broth cultures of IP2666, *ΔosmB, ΔompF,* and *ΔosmB ΔompF,* all containing the tetracycline reporter and constitutive *gfp,* were grow for 16 to 18 hours with rotation. The cultures were then diluted 1:100 and treated with doxycycline at 10ng/ml, 100ng/ml, and 1µg/ml or left untreated. The diluted cultures were incubated at 37°C for 4 hours with rotation. At 4 hours, 200µl of each culture was removed and used to detect absorbance (OD 600nm) to measure growth or density and to detect relative reporter expression by detecting mCherry emission at 610nm using a 560nm excitation to measure total in a plate reader. This same 200µl sample was pelleted at 15,000rpm in sterile microcentrifuge tubes and resuspended in 200µl of 4% PFA and fixed overnight at 4°C to be used for imaging to measure single cell fluorescence intensity.

Imaging *in vitro* Antibiotic Diffusion

To image the fixed bacterial cells, cells were immobilized onto slides using agarose pads. To make agarose pads, 1% agarose in PBS was put on a slide, covered with a square coverslip, and left to cool for 20 m. The cover slip was removed and 10µl of the fixed bacterial cells were pipetted onto the agarose pad and covered with a fresh coverslip. The bacterial cells in 4% PFA

were pelleted at 15,000rpm, the PFA was removed, and the cells were resuspended in 50µl of PBS. 4µl of the resuspension was then added onto the agarose pad. The cells on the agarose pads were then imaged at 630x magnification and images were captured using DIC and fluorescence microscopy for GFP and mCherry at the previously mentioned excitation and emission with exposures of 120ms and 13ms for the respective channels. Around 5 images were taken for each sample depending on the density of cells present on the agarose pad, the higher concentrations of antibiotics have fewer cells per frame and required more images to capture between 20 and 50 bacterial cells per frame.

Image Analysis

Images taken from the agarose pads were exported as .ZVI files to be uploaded to Volocity (manufacturer, city). To identify bacteria in each image in each channel find objects was used to identify objects. The lower size limit of objects was set at 1µm and the upper size limit at 2µm. Objects touching the edge were excluded and "separate objects" was run to identify bacteria more accurately if they were clustered together with a lower size limit of 1µm. After the images were analyzed the mean mCherry signal of each object identified was divided by the mean *gfp* signal to control for translational levels in each bacterium. These data were then imported into Prism (manufacturer, city) to graph and analyze. The data was analyzed using Kruskal-Wallis ANOVA with a Dunn's multiple comparison to determine significance.

Site-Directed Mutagenesis

To produce the M3 and M6 mutation in the tetracycline operator site (tetO) of P_{tetA}*::mCherry*-directed mutagenesis was attempted using the primer sets 5'CCACTCCCTAACAGTGTTAGAGAAAAG3' and 5'CTTTTCTCTAACACTGTTAGGGAGTGG3' for the M3 mutant, and 5'TTACCACTCCGTATCAGTGATACAGAAAAGTGA3' and 5'TCACTTTTCTGTATCACTGATACGGAGTGGTAA3' for the M6 mutant. The polymerase PfuUltra II

(Agilent Technologies, La Jolla, CA) was used to incorporate these mutations into a P_{tetA}::mCherry construct in a pMMB67EH plasmid. The following PCR cycling parameters were used: one cycle 92°C, 2 m 30 cycles of 92°C for 10s, 60°C for 20s, 68°C for 5m; and one cycle of 68°C 5 m followed by a 4°C infinite hold. After initial attempts at site directed mutagenesis were unsuccessful another attempt was made with new primer sets to incorporate the M3 and M6 mutations into both tetO sequences. The primer set 5'ATTTTTGACACTCTAACATTGTTAGAGTTATTTTACCACTCCCTAACAGTGTTAGAGAAAAG3' and 5'TTCACTTTTCTCTAACACTGTTAGGGAGTGGTAAAATAACTCTAACAATGTTAGAGTGTC3' was used for the M3 mutation, and the primer set 5'TTTGTTGACACTGTATCATTGATACAGTTATTTTACCACTCCGTATCAGTGATACAGAAAAGTGA3' and 5'ATTTCACTTTTCTGTATCACTGATACGGAGTGGTAAAATAACTGTATCAATGATACAGTGTCAACA3' was used to incorporate the M6 mutation. This was done following the same PCR cycling settings as above.

Results

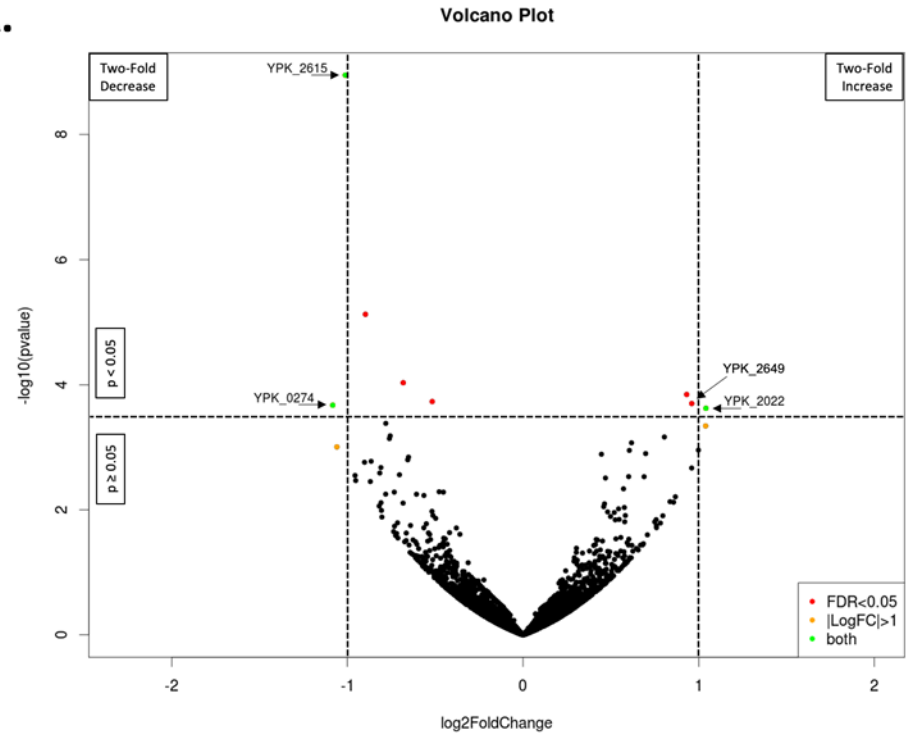
Exposure to Doxycycline Increases the Expression of *osmB* and *ompF.*

When treated with 100ng/ml of doxycycline for 2 hours, *Y. pseudotuberculosis* differentially expressed 4 genes with at least a 2-fold change and a significant P-value (Figure 4. A). The two upregulated genes were *ompF* and *osmB*, and the two downregulated genes were *tusB,* and *CNFy* (Figure 4. B). We hypothesized that these genes play a role in the development of tolerant subpopulations capable of surviving treatment with higher doses of doxycycline. Based on the location of the OsmB and OmpF in the outer membrane we hypothesized that these proteins could be affecting antibiotic accumulation and reducing the antibiotic susceptibility of *Y. pseudotuberculosis*. This thesis focuses specifically on the two upregulated genes, *ompF* and *osmB*. To determine if increased expression of *ompF* and *osmB* plays a role in the survival of *Y. pseudotuberculosis* during antibiotic treatment, deletion strains with each of the individual gene deleted, and a strain with both genes deleted, were constructed.

Effect of OsmB and OmpF on Antibiotic Accumulation *in vitro*

We hypothesized that the outer membrane associated proteins OsmB and OmpF, which are induced by exposure of exposure to 100ng/ml of doxycycline could affect tolerance to antibiotics by altering the permeability of the bacterial cell to doxycycline. If these genes alter the amount of doxycycline that is able to accumulate within an individual cell, the cell may be able to survive treatment. To determine if OsmB and OmpF altered antibiotic accumulation reporter strains with a tetracycline, inducible mCherry and a constitutive *gfp (gfp+)* were constructed for the Δ*osmB,* Δ*ompF, and* Δ*osmB* Δ*ompF* strains. The tetracycline reporter introduced into these strains was constructed by fusing the *tetA* promotor along with the repressor *tetR* from the tetracycline resistance transposon tn10 to the fluorescent protein

A.



B.

	2 h +/- Doxy comparison						
	Ascension Number	**Gene Description**	**Base Mean (Untreated)**	**Base Mean (Treated)**	**log2 Fold Change**	**Fold**	**Adjusted p-value**
Upregulation	YPK_2022	*osmB*; osmotically inducible lipoprotein B	455.775412	1199.58423	1.041	2.058	4.83E-02
	YPK_2649	*ompF*; outer membrane porin F	3998.7357	9148.47663	0.96	1.946	4.83E-02
	YPK_4157	*dut;* deoxyuridine 5'-triphosphate nucleotidohydrolase	786.140904	1714.12179	0.932	1.907	4.83E-02
Downregulation	YPK_0274	*tusB;* tRNA 2-thiouridine synthesizing protein B	1024.46974	359.333164	-1.083	2.119	4.83E-02
	YPK_2615	*CNFy*; cytotoxic necrotizing factor y	11668.7573	5488.0336	-1.01	2.02	1.82E-06
	YPK_0150	*glgA*; glycogen synthase	1252.38799	624.788023	-0.897	1.862	6.07E-03
	YPK_3445	*sodC*; superoxide dismutase (Cu-Zn)	23089.8632	13829.0637	-0.682	1.604	4.83E-02
	YPK_3822	groL; GroEL protein	98293.997	67480.2756	-0.517	1.431	4.83E-02

Figure 4. Transcriptional adaptation of *Y. pseudotuberculosis* during exposure to 100ng/ml of doxycycline.

IP2666 was incubated with 100ng/ml of doxycycline for 2h RNA was isolated and processed through RNA-seq. Transcript levels were compared to an untreated control to identify genes with an at least 2-fold change and a statistically significant change in transcript level. A) A volcano plot of the change in transcript levels in the treated population of *Y. pseudotuberculosis*. B) Altered genes with gene descriptions and transcript values for the treated and untreated samples as well as fold change and adjusted p-value. The genes that met the criteria for significant changes in expression and a 2-fold change are highlighted in red.

mCherry (Figure 5. A). In this system the repressor remains bound to the operator tetO tin the absence of antibiotics. When antibiotics are added to this system TetR becomes unbound to tetO which leads to the transcription of mCherry. This reporter allows for the approximation antibiotic accumulation within a cell. The constitutive gfp is included as a control in these strains to approximate translational activity. More metabolically active cells may be producing more mCherry in response to similar amounts of antibiotic. Measuring gfp production can help account for different metabolic rates that occur in bacterial populations and provide a more accurate measurement of antibiotic diffusion. The ΔosmB, ΔompF, and ΔosmB ΔompF reporter strains were incubated with 10ng/ml, 100ng/ml, and 1 µg/ml of doxycycline to measure the accumulation of antibiotic in each strain. Fluorescence was measured on a plate reader indicated that there was no difference at the population level in vitro (Figure 5. B-E). The mCherry fluorescence measured on the plate reader was normalized to the optical density of the culture to approximate the single cell fluorescence. The plate reader measures an average fluorescence of the entire population and measuring the population level fluorescence may not adequately represent what is occurring the single cell level.

The samples were also imaged at the single bacterial cell level to measure differences in mCherry expression. In these images mCherry signal was compared to GFP signal to ensure that mCherry signal was an accurate measurement of doxycycline exposure. This provides a relative mCherry signal to GFP signal. Analysis of the single cell image indicated that there was a significant increase in antibiotic accumulation in the Δ*ompF* strain at all concentrations of antibiotics with a dramatic increase to 10µg/ml. At 10ng/ml, Δ*osmB* did not have significantly different accumulation of antibiotics. Surprisingly, the Δ*osmB* Δ*ompF* had more antibiotic accumulation than the wild type, but significantly less accumulation than the Δ*ompF* strain

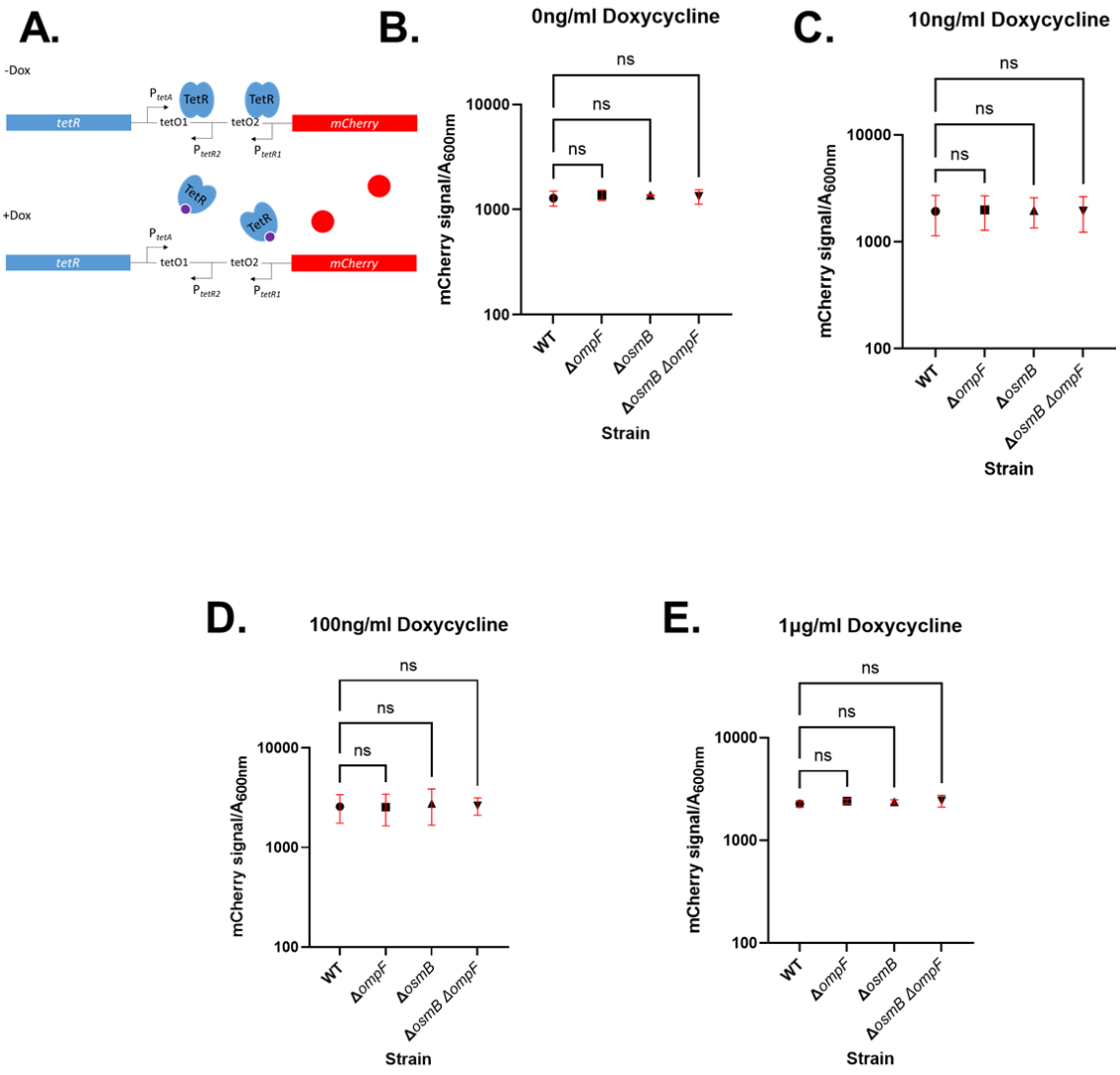


Figure 5. Population level antibiotic accumulation during treatment with clinically relevant doses of doxycycline. (A) Diagram of the reporter used to measure antibiotic accumulation based on the Tre of the tetracycline resistance gene in the Tn10 transposon. (B-E) Fluorescence from the P_{tetA}*::mCherry* reporter without treatment and with 10ng/ml, 100ng/ml, and 1µg/ml doses of doxycycline in the indicated *Y. pseudotuberculosis* strains after treatment for 4h at 37°C. Fluorescence detected by a plate reader. Fluorescent signal from mCherry was normalized to the optical density of the culture at 600nm to normalize for growth of the bacteria. The mean of three biological replicates are shown with the standard deviation. Kruskal-Wallis with uncorrected Dunn's was used to determine significance ns, not significant.

(Figure 6. B). This indicates that there may be some interaction between the two proteins. At 100ng/ml of doxycycline the Δ*ompF* strain has a lower relative mCherry value than the wild type. This is likely due to greater accumulation of doxycycline preventing the translation of mCherry in the Δ*ompF* strain. Δ*osmB* showed no difference at 100ng/ml. The slightly decreased relative mCherry expression in the Δ*osmB* Δ*ompF* strain at 100ng/ml also is likely due to greater doxycycline accumulation (Figure 6. C). At 1µg/ml all three strains had significantly higher accumulation of doxycycline than the wild type (Figure 6. D). These results indicate that there is an effect of OsmB and OmpF on doxycycline accumulation in *Y. pseudotuberculosis*. OmpF may play a more important role at lower doses of doxycycline and the increased accumulation of antibiotics in the Δ*ompF* was mitigated by the deletion of *osmB*, which may indicate that there is a relationship between the two proteins.

Role of OsmB in Survival of *Y. pseudotuberculosis* during Doxycycline Treatment in Phosphate Buffered Saline

To determine if OsmB contributes to survival during treatment with doxycycline, the survival of Δ*osmB* was to compare to the survival of IP2666 during exposure to doxycycline *in vitro*. A *gfp*+ wild type IP2666 strain and the Δ*osmB* knock out strain were used to test this possibility. Both strains were grown in the same culture during exposure to doxycycline to ensure that they were exposed to the same conditions and any differences in survival could be attributed to the absence of OsmB. The *gfp+* wild type strain was used because it contains a chloramphenicol-resistance cassette, which means that when grown in the same culture as the Δ*osmB* the wild type cells could be selected for by plating on chloramphenicol-containing media to measure growth of each strain. Both strains were grown Phosphate Buffered Saline (PBS) to determine the competitive survival. The mixed cultures were treated with 10ng/ml, 100ng/ml,

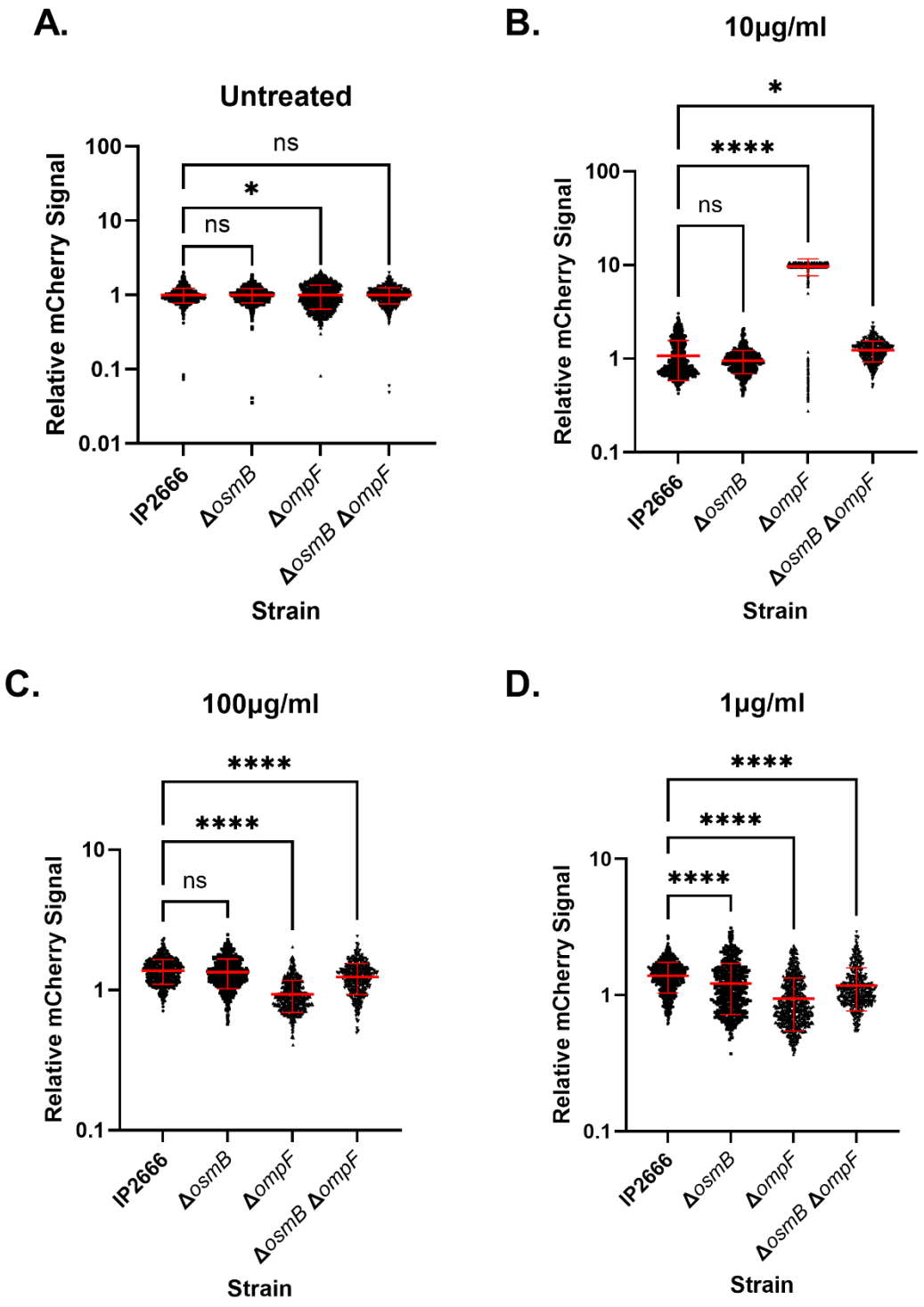


Figure 6. At the single cell level OmpF and OsmB have a significant impact on antibiotic accumulation. (A-D) Quantification of mCherry signal from the P_{tetA}*::mCherry* reporter relative to GFP signal in single *Y. pseudotuberculosis* cells in untreated cells and at 10ng/ml, 100ng/ml, and 1µg/ml doses of doxycycline in the indicated strains after treatment for 4h at 37°C. Each dot represents an individual cell. The mean and standard deviation are indicated in red bars. Kruskal-Wallis with uncorrected Dunn's was used to determine significance. *, $P < 0.05$, ****, $P < 0.0001$ ns, not significant.

and 1µg/m of doxycycline. These doses of doxycycline were used based on their previous identification as concentrations experienced by *Y. pseudotuberculosis* in microcolonies during treatment in the mouse model of infection (73). PBS was used to prevent growth during the experiment, and control for growth difference between the two strains, in an attempt to only measure antibiotic-mediated killing. We hypothesized that deletion of *osmB* would lead to less survival in the presence of antibiotics. A competitive index was used to determine if there was a survival advantage for the wild type. This was calculated by comparing the ratio of Δ*osmB* to IP2666 at 2 hours to the initial ratio of Δ*osmB* to IP2666 (mutant/WT timepoint)/(mutant/WT inoculum). The competitive indexes showed no significant difference at any concentration, indicating that the disruption of *osmB* had no impact on antibiotic tolerance in this system (Figure 7. A).

To understand why this may be the case, the total colony forming units (CFU) in the inoculum and at the 2-hour time point was counted. The difference between the inoculum and the 2-hour time point was used to determine if there was growth (ΔCFU). Without doxycycline IP2666 is capable of doubling once in PBS. When treated with doxycycline up to a concentration 10µg/ml IP2666 also was able to double (Figure 7. C). This indicated that growth in PBS for 2 hours was enough to induce a persistent phenotype in this strain. The Δ*osmB* strain also showed no significant susceptibility to doxycycline at all concentrations used (Figure 7. B). This made it difficult to determine whether there was any survival advantage for either strain and led to the redesign of the experiment. It is interesting to note that only 2 hours in a minimal media can induce such a robust persistence in *Y. pseudotuberculosis* IP2666.

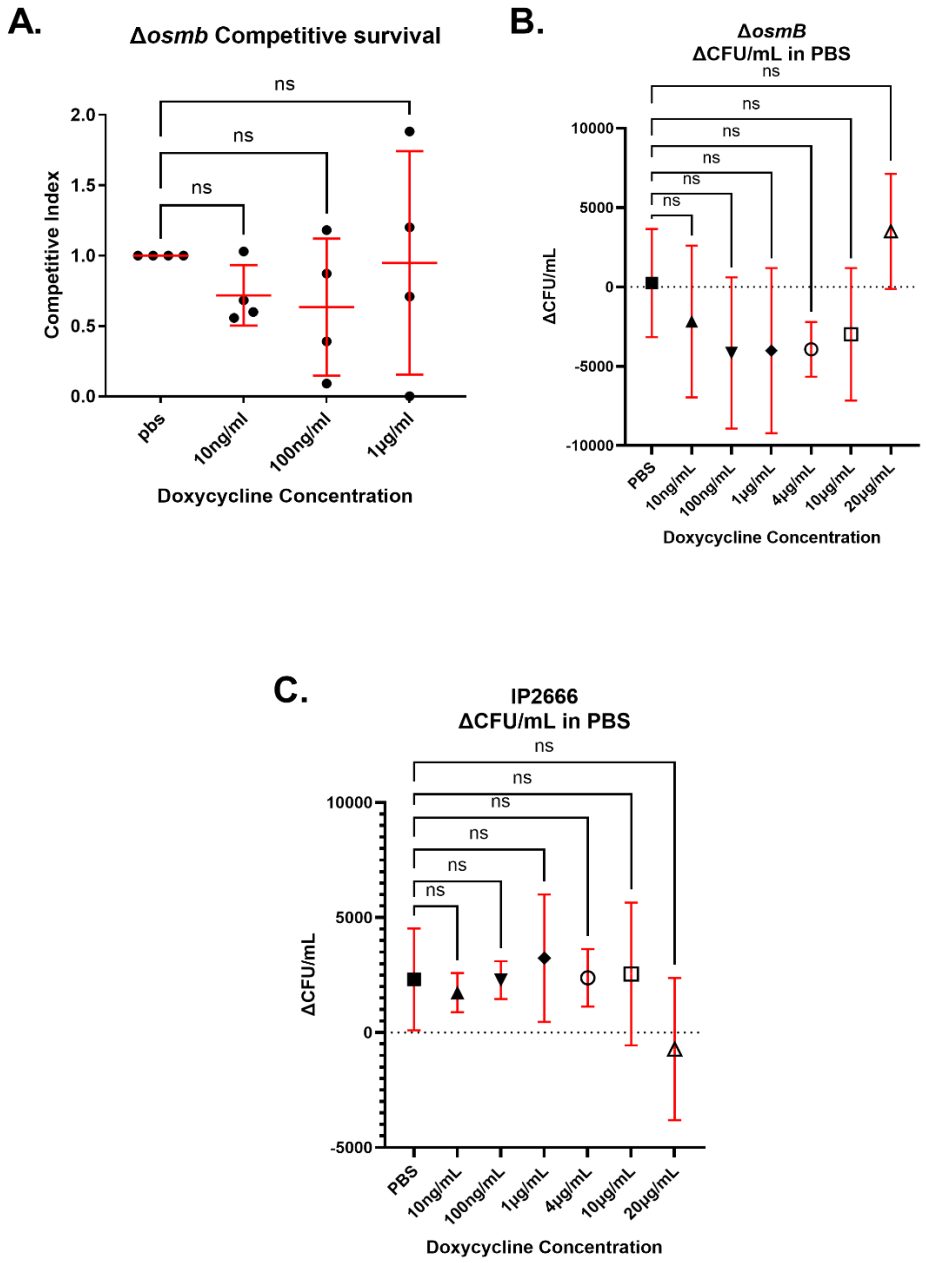


Figure 7. PBS induces tolerance to doxycycline in *Y. pseudotuberculosis*. PBS cannot be used to measure survival. (A) Competitive survival of Δ*osmB* and IP2666 after 2h of treatment with the indicated doses of doxycycline at 37°C in PBS. A competitive index was generated for each treatment condition to determine if there was a survival advantage in the WT strain. Each dot represents an individual replicate. The mean and standard error of the replicates is represented with red bars. (B) Average change in colony forming units (ΔCFU) in the Δ*osmB* strain at indicated concentrations of doxycycline after 2 hours of treatment with doxycycline in PBS at 37°C. The ΔCFU was calculated by determining the difference in CFUs at time 0 and 2h. The symbols represent the mean of 3 replicates the red bars indicate standard error. (C) Average ΔCFU in the IP2666 strain at indicated concentrations of doxycycline after 2 hours of treatment with doxycycline in PBS at 37°C. The ΔCFU was calculated by determining the difference in CFUs at time 0 and 2h. The symbols represent the mean of 3 replicates the red bars indicate standard error. Kruskal-Wallis with uncorrected Dunn's was used to determine significance. ns, not significant

Role of OsmB in Survival of *Y. pseudotuberculosis* during Doxycycline Treatment in LB

After initial experiments in PBS, the media was switched to standard bacteriological media (LB broth) to measure the effect of *osmB* more accurately on antibiotic susceptibility without inducing tolerance through growth in PBS. The concentrations of antibiotics used were also altered to focus on physiologically relevant doses of doxycycline experienced by *Y. pseudotuberculosis* in the mouse model and an inhibitory 4µg/ml dose of doxycycline. In this revised system, we would expect to see more antibiotic susceptibility and death in the Δ*osmB* strain if *osmB* plays a protective role against doxycycline in *Y. pseudotuberculosis*. Again, both the *gfp+* IP2666 strain and Δ*osmB* were incubated together in the presence of either 100ng/ml, 1µg/ml, or 4µg/ml of doxycycline and plated to measure the relative survival of each strain (Figure 8. A-D). The relative survival during doxycycline treatment was again determined by calculating a competitive index. The competitive indexes indicated that o*smB* does not affect antibiotic tolerance at 100ng/ml, 1µg/ml, of 4µg/ml. There is a great degree of variability at 4 hours in the observed competitive indexes in the 4µg/ml and 1µg/ml treatment conditions. This may be due to greater inhibition or killing reducing the sample size and leading to greater variability. There was not a significant difference in the competitive index of either strain with any of the concentrations of antibiotics used at 2 hours or 4 hours. This was unexpected as the gene was upregulated in response to 100ng/ml of doxycycline at 2 hours and there was increased doxycycline accumulation at 1µg/ml. The increased accumulation in the Δ*osmB* strain may not have been significant enough to inhibit bacterial growth.

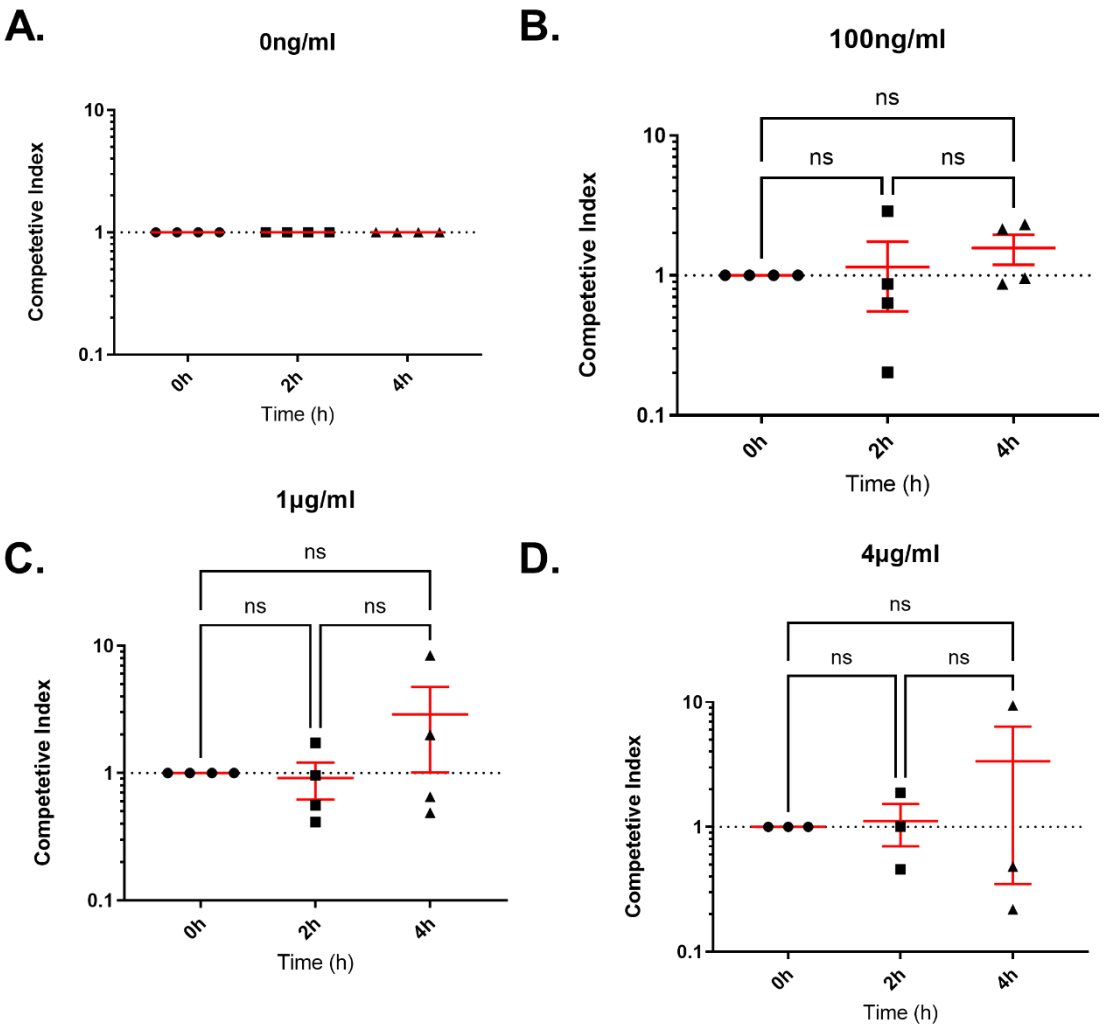


Figure 8. OsmB does not affect antibiotic susceptibility of Y. pseudotuberculosis to doxycycline at clinically relevant doses. (A) Untreated sample used to determine competitive index for treatment groups. All values are 1 for all replicates because competitive indexes are calculated by taking the ratio of the ratio of wild type to mutant in the treated to untreated. Both numbers are the same in the untreated condition, so all values are 1. (B-D) The competitive index of Δ*osmB* to IP2666 at the indicated concentrations of doxycycline. Cultures were incubated at 37°C with doxycycline for 4 hours with a sample removed and plated at 0h, 2h, and at the final 4h timepoint. Each dot represents a replicate, red bars indicate mean and standard error. Kruskal-Wallis with uncorrected Dunn's was used to determine significance. ns, not significant

Role of OsmB and OmpF in Survival of *Y. pseudotuberculosis* during Doxycycline Treatment *in vivo*

To determine if these genes had an effect on antibiotic susceptibility *in vivo* that could not be seen *in vitro,* a mouse model of infection was used. To set up for the mouse infection four strains to be used in the infection were grown overnight. The strains used were the Δ*osmB,* Δ*ompF, and* Δ*osmB* Δ*ompF* tetracycline reporter strains, and an IP2666 tetracycline reporter strain that had been used to determine antibiotic accumulation *in vitro*. Before the infection, the overnights were diluted to an estimated $1x10^3$ CFU/ml in the inoculum. Each strain was used to infect a different set of five mice through the tail veins. The mice were then treated with doxycycline 48 hours after infection to allow for *Y. pseudotuberculosis* to establish an infection in the spleen and form microcolonies. Delaying treatment also more accurately replicates a clinical setting where infected individuals would likely only seek treatment after the onset of symptoms. 24 hours after treatment with doxycycline the mice were sacrificed, and their spleens were harvested. Half of each spleen was homogenized and plated to approximate total colony forming units of *Y. pseudotuberculosis* in each spleen. This measurement provides insight into doxycycline's effectiveness at clearing *Y. pseudotuberculosis* infections for each strain. The other half of each spleen was embedded in OCT for sectioning and imaging to measure antibiotic accumulation within the microcolonies. The CUFs per spleen of the treated mice indicated that there was no significant increase in antibiotic susceptibility of the mutant strains (Figure 9.). This may be due to mouse-to-mouse variability and will need to be confirmed in a competition experiment where mice are infected with both a mutant and a wild type strain to accurately measure whether there is an effect of OmpF or OsmB on antibiotics susceptibility *in vivo*.

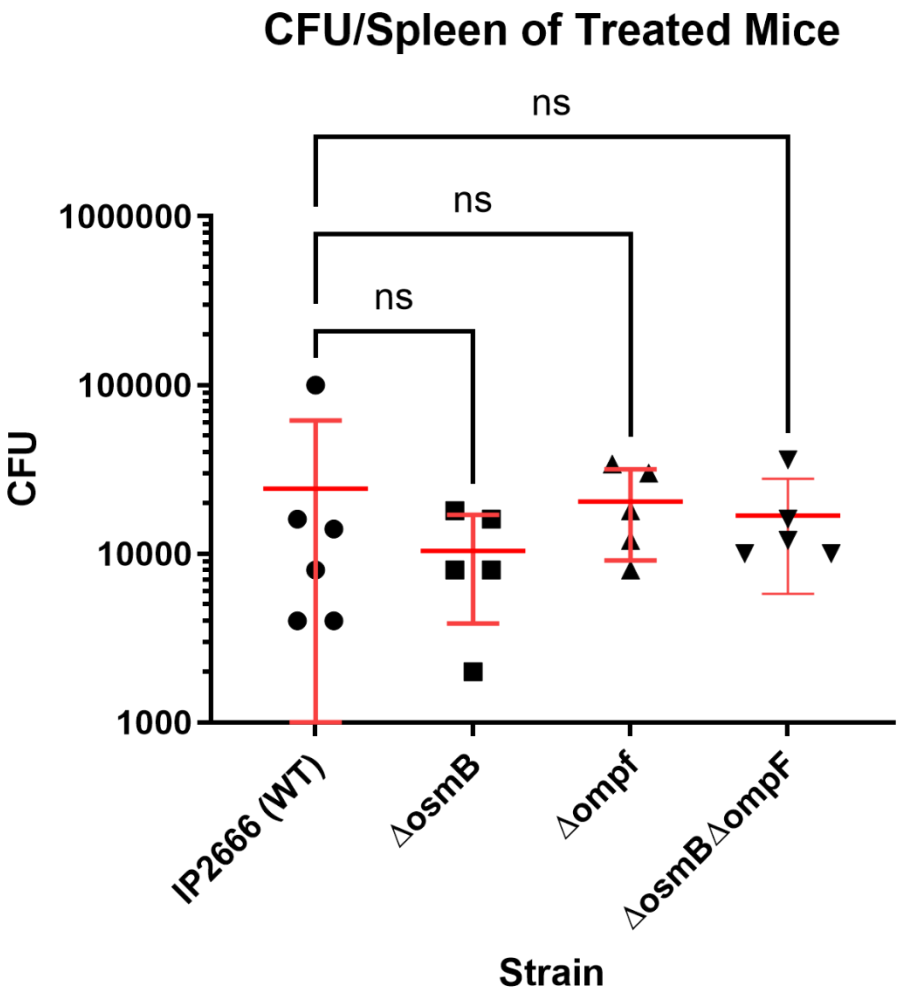


Figure 9. Single strain infections do not indicate a survival difference between Δ*osmB*, Δ*ompF*, and Δ*osmB* Δ*ompF* and WT IP2666 when treated with doxycycline.
Six- to eight-week-old C57BL/6 mice were infected with roughly 10^3 CFU/ml of each of the indicated *Y. pseudotuberculosis* strains. Mice were treated 48h after the infection with doxycycline, and spleens were harvested 24 hours after treatment to measure CFU/spleen. Each dot represents an individual mouse. This data represents CFUs quantifications from 3 individual experiments. The red bars indicate mean and standard error. Kruskal-Wallis with uncorrected Dunn's was used to determine significance. ns, not significant

Discussion

Antibiotic persistence remains a major concern in both treating infection and the continued emergence of antibiotic resistance. Establishing targets within persister or tolerant bacterial populations may allow for future development of drugs to specifically target these populations. This strategy could potentially promote the complete clearance of infections and prevent recurrent infections.

RNA-seq of *Y. pseudotuberculosis* exposed to physiologically relevant doses of doxycycline led to the Identification of four differentially expressed genes: *osmB, ompF, tusB* and *CNFy*. Further characterization of these genes to identify the role they play in persistence was carried out to determine if differential gene expression led to protection and ineffective clearance by doxycycline in our mouse model. The results of these experiments surprisingly indicated that while OmpF and OsmB have a significant impact on accumulation of antibiotics at subinhibitory and inhibitory concentrations, OsmB does not seem to contribute to survival when treated with doxycycline.

The significant impact of OmpF on antibiotic accumulation in this system is a unique finding, as it is generally thought that porins allow for influx of antibiotics. The increase in intracellular accumulation of doxycycline at physiologically relevant doses of doxycycline in the Δ*ompF* strain indicated that the presence OmpF is lowered antibiotic concentration within the cell. This result indicated that at the concentrations of doxycycline experienced in *Y. pseudotuberculosis* microcolonies during treatment, this porin is played a more important role in the passive diffusion of doxycycline out of the cell. The effects mediated by OmpF can only be accomplished through passive diffusion because it is not an efflux pump. OmpF expression may be beneficial at lower concentrations of doxycycline or when there is transient exposure to antibiotics. In these situations, the extracellular antibiotic concentration may decrease to a

lower concentration than the intracellular concentrations of antibiotics before the antibiotic is able to kill the cell. The concentration gradient could then drive diffusion of the antibiotic out of the cell through OmpF.

Other data collected in the lab indicated that there is no survival difference in the Δ*ompF* strain when treated with doxycycline. This is surprising based on the significant role that OmpF played in antibiotic accumulation at 10ng/ml and 100ng/ml of doxycycline. The lack of a survival difference, along with a difference in the antibiotic accumulation, suggests that the expression of OmpF may be beneficial during interaction with antibiotics for less than 2 hours. To determine if this is the case, additional experiments assessing potential for growth following shorter exposures to doxycycline could be performed. The OmpF deficient mutant may not be able to replicate as efficiently following treatment if doxycycline cannot exit the cell. Short-term protection may be relevant in the host where antibiotic concentrations wain after treatment and may allow for survival during transient exposure to lethal doses doxycycline.

Attempting to block OmpF as a potential drug target may not work however, because porins have previously been shown to be very important in the influx of antibiotics. This is the first demonstration of their role in the efflux of antibiotics. Porin-deficient mutants have shown increased antibiotic persistence in *E. coli* (83). The balance between targeting persistent cells and inducing persistence is a challenge, in that many proposed methods of clearing persistent infections, such as the use of ROS, are often stressors that lead to persistence.

Despite the observed increase in *osmB* expression in response to doxycycline there was little effect on antibiotic accumulation or susceptibility in when the gene was deleted. The *ΔosmB* strain showed no accumulation difference at 10ng/ml or 100ng/ml of doxycycline. However, there was slightly increased accumulation at 1µg/ml in the *ΔosmB* strain. This

indicated that while OsmB expression is induced at 100ng/ml of doxycycline, it only affects accumulation at higher lethal concentrations doxycycline. The slight mitigation of accumulation at inhibitory concentrations of doxycycline did not seem to be enough to greatly impact the survival of *Y. pseudotuberculosis*.

While there was an increased accumulation of antibiotics in the double mutant at 10ng/ml, the larger effect of OmpF seemed to be mitigated by the deletion of OsmB. This is a rather interesting result implying that there may be some interaction between these two proteins. Deletion of o*smB* may have rescued the *ΔompF* phenotype by inducing the expression of another porin such as, OmpC, but further experiments are required to assess what is occurring with this mutant and the relationship between the two proteins. OmpC and OmpF are part of a family of outer membrane proteins that allow for diffusion through the outer membrane of gram-negative bacteria. OmpC is another major porin that is that is regulated by osmolarity as with OmpF. OmpC-deficient *E. coli* mutants have been shown to be highly tolerant to antibiotics. This indicates that antibiotics diffuse into the cells through this porin, suggesting that increased expression of this porin may also facilitate outward diffusion of antibiotics and lead to the rescued phenotype in the Δ*osmB* Δ*ompF* mutant. This expression could be due to altered osmolarity in the cell because of the lack of OsmB on the outer membrane. Future work could focus on understanding this interaction as not much is known about the role of OsmB. Many lipoproteins are transduced across the outer membrane of Gram-negative bacteria through other porins in the Omp family. OmpF and OsmB may have a similar relationship; however, OmpF has not been shown to take part in this type of interaction.

It is not known if *ompF* and *osmB* are being upregulated in the same population of bacteria. The initial RNA-seq screen identified population-level gene expression patterns in response to doxycycline exposure. RNA-seq does not; however, measure transcriptional changes

at a single-cell level. The lack of single-cell data allows for the possibility that specific subpopulations could express these genes. Based on the results for the *in vitro* accumulation experiments, I would expect that these genes may be expressed by the same population at 1µg/ml of doxycycline. At this concentration both genes affect accumulation of doxycycline. At lower concentrations, between 10ng/ml and 100ng/ml, there may be two specific populations with different expression profiles for these genes; one population that only expresses OmpF and one with OsmB and OmpF expression. To understand the expression patterns within the population it will be beneficial to perform reporter experiments with OsmB and OmpF reporters both *in vivo* and *in vitro*. These strains could then be used in *in vitro* antibiotic exposure experiments to determine if the genes are expressed in the same population of cells. They could also be used in the mouse infection model to identify if there are subpopulations within microcolonies that differentially express these genes in response to antibiotics.

The rapid induction of antibiotic tolerance in the minimal media PBS was both an unexpected and highly interesting result. Within 2 hours, the nutrient deprived bacteria became tolerant to significantly higher doses of doxycycline than *Y. pseudotuberculosis* grown in LB. This rapid shift to an almost completely tolerant phenotype would be interesting to explore further. It is likely due to a shutdown of central metabolism in response to starvation, however *Y. pseudotuberculosis* can often undergo one division event after being moved into PBS. These pathways are likely similar to those utilized by other bacteria to enter a dormant state, but further elucidating these pathways in a *Y. pseudotuberculosis* model may present unique pathways or targets to understand the development of persistence. Performing similar RNA-seq-based experiments to determine which sets of genes are being expressed in response to starvation could potentially inform further experiments, including the design of new reporters, to identify bacteria that have shifted into a dormant state in an infection model within a

microcolony. If a screen identifies genes that are altered in the population of bacteria that survive treatment with doxycycline in when grown in PBS, the promotor of those genes could be fused to a fluorescent protein to generate a reporter. These reporters could be used to identify bacteria that are undergoing the same response that leads to persistence in PBS in the mouse model within a microcolony.

In vivo antibiotic susceptibility of *Y. pseudotuberculosis* WT IP2666, *ΔosmB, ΔompF,* and *ΔosmB ΔompF* in single strain infections was not significantly different 24 hours after treatment. The experiment was designed to determine if there was differential antibiotic accumulation *in vivo* and lacks data for the bacterial load at 48-hour post-infection time point when treatment was administered. This made it difficult to determine if the similar CFUs observed after treatment are the result of the same amount bacterial death from antibiotic treatment. The strains may have had growth differences in the host and replicate to higher levels before treatment with doxycycline. The unknown bacterial load before treatment makes it difficult to determine if there are significant differences in bacterial clearance when treated with doxycycline. Unpublished data from the lab suggests that the *ΔosmB* strain outperforms IP2666 in a mouse infection, so it is possible that the knockout strains significantly outgrew IP2666 prior to treatment and were then killed more efficiently. Based on mouse-to-mouse variability, these experiments should be repeated for all strains in a competition model where a mutant and wild type strain are used to infect the same mouse. This type of experiment could determine if there is increased susceptibility of the mutants to antibiotics *in vivo* and allow for a comparison in the same tissues. Using the same tissues reduces some variability because both strains are experiencing the same dose of antibiotics and are encountering the same immune response in the mouse. This is possible because the microcolonies established by *Y. pseudotuberculosis* are clonal and each would represent either the wild type or a mutant strain. The clonal colonies

allow for the comparison of two strains side by side in the same tissue. Persistence is generally thought of as a phenotype that protects the bacteria from multiple drugs so it would also be beneficial to study these genes in the context of other classes of antibiotics to determine if the accumulation differences are maintained across a broad range of antibiotics, or if it is specific to doxycycline.

Overall, the data in this thesis indicate that OmpF and OsmB significantly reduce doxycycline accumulation within *Y. pseudotuberculosis*. However, the reduced accumulation does not aid in the survival of *Y. pseudotuberculosis* when treated with clinically relevant doses of doxycycline. The identification of increased accumulation of antibiotic in the OmpF porin-deficient mutant has not previously been reported. The role of OmpF in diffusion of doxycycline out of a cell is unique and presents another possible mechanism of tolerance in bacterial species that lack multidrug efflux pumps. OsmB has not previously been associated with antibiotic persistence or pathogenicity. While the impacts on accumulation of antibiotics were not large and there was no effect on *Y. pseudotuberculosis* survival in the presence of doxycycline, insights into any potential role of OsmB are significant in that there is a very limited body of work involving this protein. The mitigation of increased antibiotic accumulation in the OmpF-deficient mutant caused by deleting *osmB* indicates that there may be some interaction between these two proteins that has not previously been reported.

In conclusion, the findings in this work to identify potential targets for the treatment of antibiotic persisters indicate that OmpF and OsmB affect antibiotic accumulation in *Y. pseudotuberculosis*. The results are clear, however, that OsmB does not affect antibiotic susceptibility at clinically relevant dose of doxycycline and targeting OsmB does not appear to be a viable method for the treatment of persister bacteria within an infection.

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